ACTA PHYSIOLOGICA SCANDINAVICA

ACTA PHYSIOLOGICA SCANDINAVICA

Vol. 72. INDEX

Fasc. 1—2. (January—February 1968.)

Fasc. 3 (March 1968)

Fasc. 4. (April 1986)

INDEX AUCTORUM



Göran Liljestrand

GÖRAN LILJESTRAND

1886—1968

After a brief illness professor emeritus Göran Liljestrand the founder and first Editor of Acta Physiologica Scandinavica, passed away on the 16th January 1968 at an age of nearly 82 years.

Liljestrand was born in Gothenburg on 16th April 1886 as the son of insurance director Erik Liljestrand and Titti Carlberg, and came to Stockholm as a youth. Early during his medical studies his scientific interest was awakened and in the Department of Physiology at the Karolinska Institute, then headed by J E. Johansson, his inclination found adequate and generous support.

After presenting his thesis in 1917 Liljestrand was appointed assistant professor (docent) in physiology in the same year and in physiology and pharmacology in 1923 When Johansson and Santesson both retired in 1927 he was appointed professor of Pharmacology H. Gertz becoming the successor of Johansson. Liljestrand retired from the chair of Pharmacology in 1951

Göran Liljestrand's name is intimately connected with the development of experimental physiology and pharmacology which began after World War I in the Scandinavian countries. Fully aware of the value of a new orientation towards the rapidly growing biological sciences in the Anglo-Saxon countries, Liljestrand at an early stage promoted and developed scientific contacts in this direction while still maintaining the traditional connections with the continental medical sciences. However the need for a Scandinavian Society for Physiology was felt increasingly more strongly as a means of coordinating and strengthening the basis for the regional development of physiological research. Following the original suggestion of S Torup in Norway this idea was strongly endorsed by Robert Tigerstedt in Helsinki and several others and was soon generally accepted.

After the first Scandinavian Congress in Physiology and Experimental Medicine, held in Lund 1925 under the chairmanship of T Thunberg, Liljestrand, as one of the founders, was assigned, together with J E. Johansson, to draft the constitution of the new Society which met in the following year in Stockholm in connection with the International Physiological Congress. Liljestrand who was General Secretary of the Congress also became the first secretary of the Society serving in this capacity 1926—1948. As a token of appreciation for his valuable work he was elected honorary member of the Society in 1951 A commendable historical account of the Society was given in Turku 1966 by C. G Bernhard on the occasion of its 40th anniversary

In the 1930ies it became increasingly clear to Liljestrand that the situation with regard to the publication of papers within the field of physiological sciences in the Scandinavian countries was not satisfactory. For many years a great proportion of such communications had been published in Skandinavisches Archiv für Physiologie, owned by a German Publisher and with professor C. G. Santesson as Editor-in-chief. To a certain extent the publication of many articles in the German language hampered their access to the Anglo-Saxon readers, but an even more important reason to seek a change was the rapidly deteriorating political situation, which made it undesirable that the Scandinavian papers should appear in a Journal, which by its very name implied a greater degree of connection or even dependence than actually existed. For this reason Liljestrand, with the full support of his Scandinavian colleagues, was determined to found a new Journal as an organ for the Scandinavian Society for Physiology and also became its first Editor-in-chief (1940) aided by representatives for Denmark (A. Krogh), Finland (Y. Reenpää) and Norway (E. Langfeldt). This also meant, of course, that the old ties with the German Publisher had to be broken, which—understandably—was not left unnoticed by the German foreign relations representatives. The decision was made, however, and a rearrangement of this kind was due to occur sooner or later under any circumstances.

Liljestrand's eminent competence and unfailing efforts have been instrumental in building up and consolidating the new Journal, of which he remained as Editor until 1957. Nordic Physiology owes him a large debt of gratitude for his achievements in this area.

Liljestrand was a faithful attendant at the Meetings of the Scandinavian Society for Physiology as well as of the local Physiological Society in Stockholm. His influence in physiology in Scandinavia as a whole and in his own country in particular was excercised in a variety of ways. The students agreed that he was an excellent and entertaining lecturer who often injected notes of historical or personal interest, not infrequently in anecdotical form. In the Physiology Meetings he not only gave well-formulated talks about his own research but also appeared in the discussions in variety of subjects with great élan. He had an unusually clear mind and with remarks and unshakable logic he more often than not scored victories in the

It was only natural that Liljestrand's competence should be utilized in many fields. Thus he served as secretary in the Nobel Committee for Physiology or Medicine 1918—1960 and was also member of the Committee 1938—1950. In the Nobel Foundation he was trustee 1945—1966 and acted as chairman of the trustees for many years. In the Royal Academy of Sciences he took a very active part and acted as President in 1951—1952. As secretary of The Swedish Society for Medical Research he aided many young research students, and in the Physiological Society in Stockholm he became honorary member in 1951 in recognition of his effective work in this Society as founder and member. The importance of international scientific contacts was always clearly recognized by Liljestrand. Consequently he took great interest in the international congresses of physiology and was a member

of the permanent committee for many years. In all of these capacities Liljestrand exerted his wide knowledge, his great experience and his capacity to solve problems for the benefit of the organization to which his interest was devoted.

As a Department Head and leader of young scientists at a time when the whole staff consisted of himself a junior assistant and a department attendant, Liljestrand guided a great number of young scientists during their initial stages of research. He complemented his own educational skill by arranging for his graduate and post graduate students to go abroad to well appointed laboratories for further training.

Throughout his active life Liljestrand dedicated a large part of his time to research in physiology and pharmacology. His interest was first directed chiefly to respiration and respiratory gas exchange during work and various other conditions. In a careful study on ventilation during artificial respiration in man, together with G. Wollin and J. O. Nilsson (1915) the authors demonstrated the hazards of measuring ventilation in "passive" subjects since these as a rule respire involuntarily. During apnoea the ventilation effect was consequently considerably less than without apnoea. The low efficiency of the ordinarily used methods no doubt encouraged the efforts to find better techniques for artificial respiration.

Liljestrand' interest in ventilation was also expressed in the subject for his Thesis "Untersuchungen über die Atmungsarbeit" published in 1917. The main result of this work was the demonstration that the ventilatory work during normal respiration only amounted to 1—3 per cent of the total basal metabolism instead of 10—15 per cent as usually believed at that time. Voluntary hyperventilation made ventilation less economical. By introduction of additional dead space a convenient method for varying the pCO_2 and ventilation was obtained.

Liljestrand's clear style and his profound knowledge of the literature made him particularly suited to write reviews and surveys. A model article of this kind was his "Physiologie der Blutgase" from 1928, published in the famous Bethe—Bergmann "Handbuch der normalen und pathologischen Physiologie".

In the 20-ies Liljestrand devoted most of his research work to problems concerning the influence of various factors on the basal metabolism and cardiac out put. The nitrous oxide method introduced by Krogh and Lindhard and the simple technique for measuring oxygen consumption, described by Krogh, opened up new fields of study and the close connections and friendship between Liljestrand and the Danish Physiologists no doubt greatly promoted these studies.

A series of papers with N. Stenström (1917—1925) deal with the metabolic rate during swimming and other types of exercises.

Liljestrand was early attracted by the possibility of finding a formula by which the cardiac output could be expressed, using more easily obtained parameters such as heart rate and blood pressure. With Zander he published in 1928 the well-known frequency reduced-amplitude-product formula which gives a very useful indication of the relative cardiac output.

In addition to a study on the "heat puncture" in rabbits (with K. Frumerie) and a study on tetanus muscle contracture, the result of his stay as a research student in

Rudolf Magnus laboratory in Utrecht, Liljestrand published a paper "Über den Schwellenwert des sauren Geschmacks" (1922) in which he could show by using buffered solutions, that the threshold for acid taste is about pH 6 and not pH 3—4 as found with unbuffered solutions.

During this period he also spent some time abroad as a research student in J. Barcroft's laboratory in Cambridge and with A. V. Hill in London.

The writer of these lines had the privilege of working as a graduate student in Liljestrand's laboratory 1926—1939. It is hard to imagine a more helpful and stimulating teacher and co-worker whose deep knowledge and imaginative thoughts always gave new aspects to the problem.

Heymans' discovery around 1930 of the chemoreceptor reflexes governing respiration, and to some extent also circulation, profoundly changed the outlook in these fields. Liljestrand at once recognized the significance of these findings, and in an extensive series of papers in the 30-ies he took up this new line of research, in co-operation with Zotterman in the first place. From this fruitful co-operation, in which I had the privilege to take some part, a number of results were obtained in which Zotterman's experimental skill and experience with recording action potentials from single or small groups of nerve fibres were of greatest significance. By correlating the activity in the sinus nerve with the composition of the gas breathed the chemoreceptor activity could be put on a quantitative basis. A number of drugs were tested by the same technique, particularly in the later phase of the work in which S. Landgren cooperated. It was not surprising that Liljestrand should chose this topic for the address delivered at the Royal Academy of Sciences on leaving the chair as president of the Academy in 1952.

During these joint efforts Liljestrand offered numerous valuable suggestions and ideas which he pursued with great perseverance. In particular he stressed the importance of acetylcholine as transmitter in the carotid body and also a change in pH as a common denominator in the mechanism of action of carbon dioxide, anoxia, and various metabolic inhibitors. Thus, at a meeting in Philadelphia following the International Congress in Montreal 1953 he lectured on transmission at chemoreceptors. His concept of a peripheral "afferent" chemotransmitter mechanism was applied to the taste receptors, and again the results suggested that acetylcholine is involved in the transmission process. These studies may well prove to have greater significance than recognized at the time.

The importance of the sinus mechanism for the blood pressure homeostasis during muscular work was also clearly demonstrated in a paper published in 1946.

Liljestrand's agile mind also turned to the old question of pulmonary circulatory regulation, and in a joint study in 1945—1946 we were able to show that oxygen lack as well as excess carbon dioxide raised the pulmonary arterial blood pressure in the spontaneously breathing cat. These results aroused considerable interest and have also been confirmed in man. Liljestrand at once saw the important physiological implication of this unusual response—which occurs in the reversed form in the umbilical vessels and placenta—and concluded that the direct vasoconstrictor effect

of oxygen lack and carbon dioxide on the pulmonary vessels constituted an important mechanism, by which the pulmonary blood stream was directed towards the better ventilated parts of the lung.

At the age of 76 Liljestrand still took part in active experimental work on the effects of posture on alveolar—arterial CO_2 and O_2 differences and on alveolar dead space in man with H. Bjurstedt, C. M. Hesser and G. Matell, showing his unconquerable interest in research. At the Scandinavian Congress of Physiology in Turku 1966 he gave a much appreciated paper on distribution of the pulmonary blood flow when he was 80 years old, and although his voice was not as strong as earlier he still presented the paper in his usual brilliant way.

During all his life Liljestrand was a convinced temperance man and made many valuable contributions, particularly in connection with the hazards of drunken driving. However his tolerance and wisdom never let his personal views as regards the use of alcoholic drinks influence his attitude in social reunions with friends and colleagues who did not follow equally rigid rules. As one might expect, Liljestrand also fought energetically against quackery and homeopathy in a number of well written articles.

Liljestrand's interest in the history of medicine is witnessed by a number of publications. In addition to several articles on various subjects, he has published together with B. Holmstedt "Readings in Pharmacology" (1963) which is a goldmine for those who are interested in the history and development of pharmacology and of remedies. A profound knowledge of the subject, aided in many cases by previously unnoticed material from early documents makes this volume stimulating and entertaining reading.

Of special interest for Scandinavian scientists is Liljestrand's article on Paul Sjöquist and insulin, in which the experiments of the Swedish doctor are brought into the light. As pointed out by Liljestrand, the results might have been successful, if the basic conditions for experimental work in this field had been more favourable.

Liljestrand had the great satisfaction last year at 81 to see his paper on Carl Koller and the development of local anesthesia in print as a supplement in this Journal. This paper is a remarkable proof of his unimpaired mental capacity and even apart from its great historical value, it provides a most engaging reading and has much of the qualities and suspense of a good criminal novel.

His masterful use of the pen is also evident from a large number of articles on various subjects in a variety of fields. It is not possible to list these products of his diligent hand, but at least his excellent prefatory chapter in Annual Review of Physiology 1957 "The Increasing Responsibility of the Physiological Sciences" should be mentioned. In this thought-provoking essay Liljestrand found an occasion to put forward some of the ideas and aims he had himself long been working for.

Liljestrand's lively and incessant interest in the training of new generations of scientists is also illustrated by his address at the inauguration ceremony of the newly created doctors in Stockholm 1967 when he himself received the insignia of a jubilee doctor 50 years after the presentation of his thesis.

The impact and influence of a scientific personality is not always possible to appreciate at the time of his death. However in the case of Göran Liljestrand there is no doubt whatever that he has played a very important role for the development of experimental research in the physiological and pharmacological sciences in his country. His wide contacts and inspiring guidance of young scientists his open mind and his clear insight that research requires support for the experiments as well as *for the research worker has secured him a place of honour in Scandinavian Medical* Sciences. He shall be remembered with respect, admiration and gratitude.

Liljestrand had a delightfully simple and friendly way with young and old, abhorring any form of conceit and pompousness. His helpful and generous mind in connection with his profound knowledge and keen-witted conversation made him an unforgettable colleague and friend.

ULF S. VON EULER

Reproduktion of bronze plaque by Gustaf Nordahl 1951 Photo J. Wahlbärj.

Acta physiol. scand. 1968, 72. 1—5

From the Institute of Physiology and Medical Biophysics, University of Uppsala, Sweden

Simulator for Demonstration of Some Bioelectric Properties of Biological Transducers

By

P. Å. Öberg and U. Sjöstrand

Received 20 February 1967

Abstract

Öberg P. Å. and U. Sjöstrand. *Simulator for demonstration of some bioelectric properties of "biological transducers"*. Acta physiol. scand. 1968. 72 1—5

An electronic simulator demonstrating some of the properties of biological transducers is described. It is possible to simulate the "nerve activity" and its relation to "generator potential" and the "adaptation phenomenon" for some different kinds of biological receptors.

As part of the instruction in sensory physiology there is a need to demonstrate the bioelectric properties of biological transducers, for example the baroreceptors. In a biological preparation, it may appear difficult to demonstrate both the graded generator potential response of the receptor organ, and, simultaneously the spike discharge of the nerve axon resulting from the generator potential. We therefore thought it worthwhile constructing a simple simulator to demonstrate the dynamic properties (from a bioelectric point of view) of some "biological transducers".

The principle of the simulator is shown in the diagram (Fig. 1 (a)). It contains a relaxation oscillator circuit utilizing a unijunction transistor 2N2646 (General Electric Co., New York, U.S.A.) This type of transistor has "dynatron" characteristics Fig. 1 (b) i.e. V I-diagram has an interval with negative resistance. The

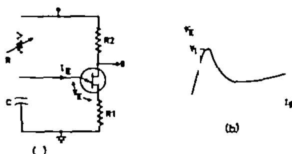


Fig. 1 ( ). Equivalent diagram and (b) V I-properties according to the text.

Fig — Circuit diagram.

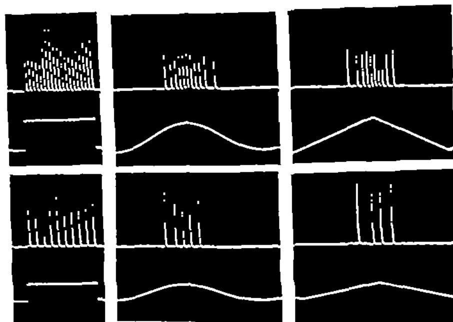


Fig 3 "Spike discharge" (above) for various changes in the "generator potential" (below) depolarisation in both recordings upwards

capacitor C is charged through the resistance R. When the potential (V_E) has reached the value V the transistor conducts, so that the capacitor is discharged through the resistance R_1 at the same time as current flows in the circuit R_T—R and a pulse ("spike discharge") is therefore obtained at O in relation to ground. The frequency of the relaxation oscillator depends on the value of R. In the present wiring diagram, R is controlled electronically by the input signal ("generator potential") The complete circuit diagram is shown in Fig 2. The necessary negative input signal is obtained from a function generator and both this signal and the output signal from the unijunction transistor circuit is recorded on an oscilloscope. There is also an output facility for negative spike discharge.

Fig. 3 shows the "spike discharge" (upper trace) for various changes in the "generator potential" (lower trace depolarisation upwards) similar to the well known pulse-position modulation in the biological receptor preparation (in the case of no adaptation properties)

From a "threshold" level in stimulus and up to a certain saturation level, there is a linear relationship in the response of the biological receptor preparation between the size of the generator potential and the impulse rate in the afferent nerve axon (*cf* Fuortes, 1958) These properties are also to be found in the simulator described here (Fig. 4) Analogous formulae for the electrokinetics of mechanoelectric transduction have been shown by Teorell (1966) with a linear relationship between intensity of stimulation ("pressure") and "spike frequency"

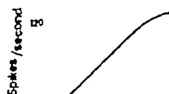


Fig. 4. Size of "generator potential" and "spike discharge frequency in the simulator described in the text.

The adaptation phenomenon, known for many different kinds of biological transducers, can also be demonstrated with this simulator. By introducing a time-varying, nonlinear element (Raysistor CK 1121 Raytheon Co. Newton, Mass. U.S.A.) in a feedback loop in the amplifier of the simulator the adaptation of different kinds of biological transducers can easily be demonstrated. Four types of biological transducers (Fig. 5) described in the literature (*cf.* Adrian, 1928) are choosen to show different adaptation rates (obtained by switch O in Fig. 2).

The skewness cannot be demonstrated with this simulator, i.e. different sensitivities for positive and negative changes in stimulus and postexcitatory depression phenomenon described for the baroreceptors of the carotid sinus (Landgren, 1952) and the muscle spindles (Adrian and Zotterman, 1926). These mentioned properties

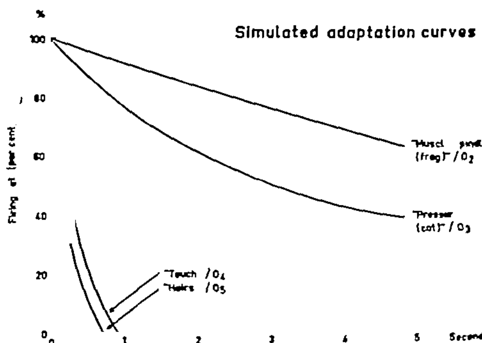


Fig. 5. Simulation of adaptation in different biological receptors as a response to a constant stimulus. O2, O3, O4 and O5 indicate positions of switch O in Fig. 2.

can, however be added to the simulator. A more elaborate simulator having the properties of the carotid sinus baroreceptor mechanism, is presently used in dog experiments and will be published later.

Financial support for this investigation was provided by Magnus Bergvall' Foundation, Stockholm, and the Medical Faculty of the University of Uppsala, Uppsala, Sweden.

References

Adrian, E. D. *The Basis of Sensation*. New York 1928. 79.

Adrian, E. D. and Y. Zotterman, The impulses produced by sensory nerve endings. *J. Physiol. (Lond.)* 1926. *61*: 151—171.

Fuortes, M. G. F. Electrical activity of cells in the eye of Limulus. *Amer. J. Ophthal.* 1958. *46*: 210—223.

Landgren, S., On the excitation mechanism of the carotid baroceptors. *Acta physiol. scand.* 1952. *26*: 1—34.

Teorell, T. Electrokinetic considerations of mechanoelectrical transduction. *Ann. N. Y. Acad. Sci.* 1966. *137*: 950—966.

Acta physiol. scand. 1968. 72. 6—14

From the Institute of Neurophysiology University of Oslo and Submarine Command
Royal Norwegian Navy Norway

Lung Damage and Lethality by Underwater Detonations

By

P Andersen and S. Loken

Received 3 April 1967

Abstract

Andersen P and S Loken. *Lung damage and lethality by underwater detonations.* Acta physiol. scand. 1968 72. 6—14

Adult anesthetized rats were immersed to the larynx and subjected to underwater blast from two charges of 1 g tetryl each, both set off at a distance of 0.8 m from the target. The ratio weight of the damaged lung/weight of the normal lung expressing the degree of the lung hemorrhage and the lethality (one hour survival) were both larger when the two charges were separated by a time interval of 8—25 μsec as compared to simultaneous detonation. The results indicate that the time integral of the pressure is more important for underwater blast damage than is the peak pressure attained.

The damage produced by an underwater detonating charge is due to the pressure ve crossing the interphase between tissues with specific gravity close to that of g on one hand and gas-filled cavities on the other Hence the greatest damage l occur in the lungs, and at the tissue/gas interphase in the gut (Zuckerman 40 Wakeley 1945)

The pressure pulses produced by an underwater charge are illustrated in Fig 1 An initial sharp pressure peak is followed by a subsidiary pulse (SP) The charge is transformed to a sphere of gas which expand and later is compressed due to the elasticity of the water At the end of the phase of compression of the newly formed gas a bubble pulse (BP) appears, shortly afterwards followed by a second subsidiary pulse (SP) A single detonation gives a very fast initial rise of the water pressure to a maximal value (p_{max}) whereafter the pressure falls exponentially with a time constant T given by $p_T = \frac{1}{e} p_{max}$

Fig 1A shows the two types of reflexions experienced by a submerged target (diver) Provided the bottom is sufficiently hard, the pressure wave is reflected from it with a positive sign forming a bottom reflection (br) which adds to the pressure (Fig. 1C, middl The pressure wave is also reflected from the surface This surface reflection (Fig 1A sr) has a negative sign and reduces the existing

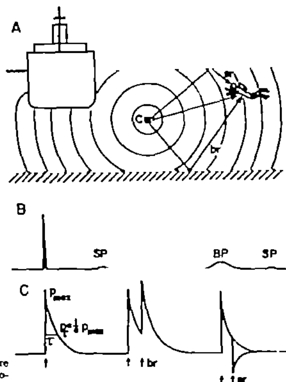


Fig. 1 Diagram explaining the pressure waves produced by an underwater detonation.

pressure on the target (Fig. 1C, right) The surface reflection can be visualized as a process which disrupts the upper horizontal water lamellae by tearing them away from the surface. It is responsible for the "boiling that appears very soon after the detonation of a submerged charge

The mechanism leading to the damage produced by an underwater detonation is not fully understood. Barcroft (1939) maintained that the lung damage was due to an expansion of the lungs with subsequent tearing of the organ. However Zuckerman (1940 1941) and Krohn, Whitteridge and Zuckerman (1942) showed that the damage developed as soon as the pressure wave hit the body Later this theory has been substantiated by experiments by Bebb (1954) and in a series of investigations into the effect of air blasts by Clemedson and his associates (Clemedson, Hultman and Grönberg 1953, Clemedson 1954 Clemedson and Hultman 1954 Celander *et al* 1955, Clemedson and Criborn 1955)

According to Bebb (1954) experiments on *on-living targets* have shown the impulse of the pressure wave passing through a given area of water to be a dominating factor for the degree of the damage. This is true provided the natural period of the target is considerably longer than the time constant of the pressure wave If, however the time constant of the pressure wave is considerably longer than the natural period of the target, the main factor is the maximal pressure

TABLE I Lung weight of albino rats

Lung	n	per cent of body weight	
left	34	0.261	0.07
right	34	0.216	0.0r

The natural period of the lungs is not known. It is, therefore difficult to conclude from the available evidence which parameter is of the greatest importance in producing lesions in submerged living beings.

The aim of the present investigation has been to determine whether the peak pressure or the time integral of the pressure is the dominant factor in the damage produced by underwater detonations. Furthermore the effect of the anaesthetic level on the degree of damage was studied.

Methods

Albino rats (120—250 g) were anesthetized with sodium pentobarbital, 25 mg/kg body weight. I initial experiments, the relation between the weights of the right and left lung respectively t the body weight were determined. The results are given in Table I. After anesthesia, the thorax was opened and the lungs removed. Each lung was weighed after isolation and gentle drying of the surface with absorbing paper.

I the detonation experiments, the anesthetized rats were suspended from a wooden frame floating in the water (Fig. 2). The fore- and hindlegs were tied t a steel frame which hung from a beam across the wooden frame. The steel frame kept the rats in ertical position with the entral side tow rds the charge, to maintain constant distance of 0.8 m from the

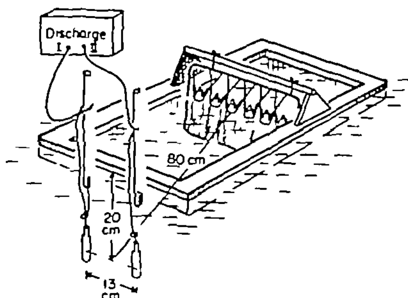


Fig. 2. Diagram showing experimental rrangement. The animals re kept in steel frame submerged to the larynx. Two 1 g tetryl harges were set off with different delay by he detonator box.

midpoint between the tw employed charges to all six rats of each series. The rats were pr treated ith wetting agent to remov any air bubbles in the fur Care was taken to have all animals submerged to the same extent, with the larynx at the water surface.

The charges were two similar electrical detonators, ICI nr 8, each containing 1 g tetryl. Initial experiments showed that there had to be a minimal distance of 7 cm to avoid simultaneous detonation when one charge only was set off. The two charges were fastened to two ertical steel bars and placed 13 cm from each other 20 cm under the water surface. Using an electronic discharge mechanism, the two charges were detonated with time delays of 0 8, 15 20, 25 60 600 and 2000 μsec. To avoid reflexes from nearby structures, the detonations were performed in open water

The degree of the lesions were measured according to the methods advised by Clemedson (1949) The damaged lungs were weighed, and the lesion expressed by the ratio between the observed lung weight and the normal weight of the lung in question. The degree of emphysema was judged subjectively The lethality was taken as the number of dead animals one hour after the detonation.

The animals were divided into four groups according to their depth of anesthesia, judged from their behaviour:

Group 1 Spontaneous small movements and occasional ocalization.
Group 2 Crying on pinching of the skin.
Group 3 Weak reaction to pinching of the skin.
Group 4 N reaction to severe pinching of the skin.

Results

Lung damage The lung damage consisted of bleeding and emphysema. In all cases, the right lung suffered greater damage than the left lung. The basal areas were more often damaged than the apical parts, and also the entral margins more frequently than the dorsal ones. In the present experiments, pneumo- or hematothorax, or rupture of the pleura or the diaphragm were not observed. Hemorrhages indicating concussion of the brain and spinal cord were frequently noted.

When heavy lung damage occurred, the bleedings were as a rule confluent, often with sharp delineations, indicating that the blood had entered a large bronchus and filled the pulmonary alveoli retrogradely The bleeding was assessed by the ratio between the weights of the lesioned (W) and normal lungs (W)

The degree of the lung damage was dependent upon the delay between the detonation of the two charges. As seen from Fig. 3 and Table II a short delay between the two charges increased the damage as compared with simultaneous de-

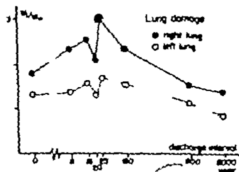


Fig 3 The lung damage (W/W ratio) is plotted as function of delay between tw successive underwater discharges. Encircled line indicates statistical significance (p < 0.01)

TABLE II Lung damage

Number of detonators	Time delay µsec	Right lung W_1/W		σ	Left lung W_1/W	n	
2	0	1.80	10	0.61	1.34	10	0.44
2	8	2.34	12	0.27	1.37	12	0.27
2	15	2.55	18	0 77	1.58	18	0.46
2	20	2.10	12	0 70	1.32	12	0.45
2	25	3 01	12	0.70	1 70	12	0.34
2	60	1.99	12	0.60	1.32	12	0 41
2	600	1.53	14	0.30	1 13	14	0 38
2	2000	1.37	6	0.22	0 83	6	0.11
1	—	1.51	22	0.45	0.96	22	0.20

tonation The most marked increase of the damage was found at discharge intervals from 8—60 µsec. The dip in the curve corresponding to a discharge interval of 20 µsec can probably be explained by a somewhat lighter anaesthesia of these animals. Discharge intervals beyond 2000 µsec did not give additional lesions.

A simultaneous detonation of two charges resulted in a 34 per cent increase of the W /W ratio for the left and an 80 per cent increase for the right lung The increase of the p_{max} above the peak pressure of the first pressure wave, would only be 26 per cent. However separation of the two charges by 25 µsec gave a marked ase of the W_1/W ratios, amounting to 70 and 200 per cent for the left and lung respectively

In addition to the bleeding, the lung damage was manifested by oedema This condition wa often marked, and was sometimes observed without concomitant microscopical bleeding Our impression was that the oedema was the most important lethal cause in those animals that died immediately Many animals which were dead when taken out of the water had massive lung oedema with small or no bleeding at all. In the survi ing animals, the oedema disappeared rapidly Thus, two animals, apparentl moribund were llowed to recover and were both in relatively good shape after s x hours At subsequent a topsy the lungs were microscopically almost normal, v ith mall subpleural hemorrhages only

The small degree of damage of the left lung compared to that of the right side was striking In the left l ng the bleeding was always found along the marginal areas of the lungs, ery often in the cardiac lobe

Lethality The lethal effect also aried as a function of the interval between the detonation of the two charges (Fig 4 and Table III) The effect on the lethality was very similar to that on the l ngs Applying a statistical limit of P<0 01 three interdetonation values, 8, 15 and 5 µsec gave ignificantly increased lethality

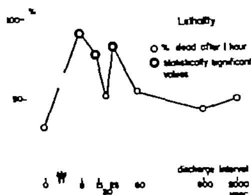


Fig. 4 Lethality (1 hour survival) plotted against delay between tw ccessive discharges. Encircled values are statistically significant ($p<0.01$)

TABLE III Lethality

Number of detonators	Time delay µsec	n	Dead	
			number	
2	0	10	3	30
2	6	12	11	92
2	15	18	14	78
2	20	12	6	50
2	25	12	10	83
2	60	13	7	54
2	600	14	6	43
2	2000	6	3	50
1	—	34	19	55

C rrel tion between d g e of anesthesia d the severity of les as In Fig 5 the importance of the depth of anesthesia on lethality (A) and on the lung damage (B) is illustrated. The results indicate that both the lethality and the lung damage increase w th deeper levels of anesthesia. The correlation is clear with regard to lethality whereas it is only suggestive when the degree of lung damage is calculated. The explanation might possibly be sought in the diminished ventilation during deep anesthesia

Discussion

Nature of lesion In accord with earlier investigations (Wakeley 1945 Bebb 1954) the most freq ently observed biological lesions were lung bleeding and oedema, and rupture of the gut w th peritoneal bleeding. The main damage is due to the rare faction wav that is created when th pressure wa e tra els from tissue of high density into a cav ty of gas with much lower density However in addition to the gas,

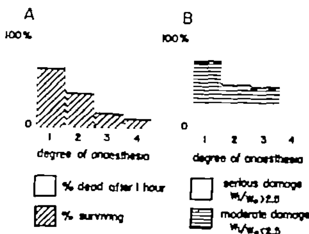


Fig. 5. Diagram over relation between depth of anesthesia and lethality (A) and lung damage (B). The degrees of anesthesia 1—4 are defined in Methods.

water interphase lesions, reported by other authors, considerable damage was done by concussion of the brain and spinal cord, as judged by localized bleedings and the function.

Parameters of pressure wave responsible fo biological damage. Experimentally the time integral of the pressure I is gi en by the equation

$$I = p\,dt \qquad (1)$$

has been found to be proportional to $\frac{M}{d}$, where *M* is the mass of the charge and *d* is the distance between the charge and the target (Wakeley 1945). The numerical value of I is dependent upon the type of explosive used. The pressure *p* at a time t r detonation is gi en by

$$p_t = p_{max}\, e^{-} \qquad (2)$$

here *τ* *is the time constant of the pressure curve. The water pressure is found to* be proportional to $\frac{M}{d}$. The energy transmitted to the water by the charge is proportional to $\frac{M^{\frac{1}{}}}{d}$. The results of the present investigation indicate that the peak pressure is not the sole factor responsible for the biological damage. The experimental data presented supported the iews expressed by Wakeley (1945) and Bebb (1954) from experience with human cases. In addition to the maximal pressure obtained, the duration of the overpressure seems to be of great importance. In Fig. 6 is plotted the time course of the damage to the right lung (W/W ratio) with the calculated time integral of the pressure and the maximal pressure calculated for two 1 g charges being detonated at the indicated time intervals. The maximal pressure is largest at simultaneous detonation *and falls within 15 μsec to the level* attained by a single discharge. The time integral curve for the pres ure increases

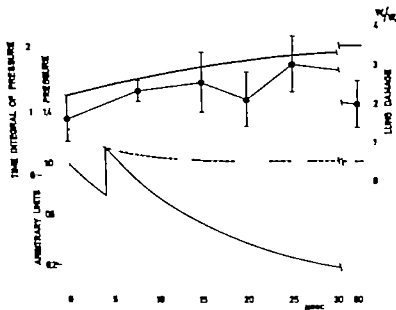


Fig. 6. Plotting of peak pressure (broken line), time integral of pressure (full line) and lung damage (filled circles) as functions of the separation of two similar underwater discharges. The lower line describes the pressure curve which would be produced by two detonations of 1 g tetryl each, set off with a time interval of 4 µsec. The peak pressures and the time integral of the pressure are calculated, whereas the lung damage values are experimental.

steadily and obtains its maximum about 40 µsec later (4). The initial part of the pressure integral curve coincides with the plotting of the increased lung damage and the lethality. This correlation applies where the charges are separated by a period roughly corresponding to twice the time constant of the pressure curve produced by a single explosion. These data suggest that the biological damage is closely related to the time integral of the pressure curve.

With regard to the relative importance of the maximal pressure and the time pressure integral, certain deductions can be made. As seen from Fig. 3 and Table II, the amount of lung damage assessed by the weight ratio damaged lung/normal lung increased by 20 per cent when two charges were detonated instead of one. The maximal pressure produced by two charges is 26 per cent larger than that of a single discharge. The time pressure integral is also 26 per cent larger than that due to a single detonation. The results obtained by separating the two detonations by 25 µsec were very different. In this situation, the lung damage was about 200 per cent of the damage elicited by a single discharge, whereas the maximal pressure should only increase 14 per cent above the single discharge value. On the other hand, the time pressure integral calculated to three times the single discharge time constant increased by 40 per cent. The very large increase in the lung damage and lethality in spite of a moderate reduction of the maximal pressure suggests that the duration of the overpressure is the main factor involved in the biological damage. This is

different from the conclusion reached from air blast experiments (Celander *et al.* 1955) in which the magnitude of the static overpressure was of greater importance than the duration of the overpressure. However the energy delivery of an air pressure wave differs from that of a pressure wave in water. A large fraction of the energy contained in an air pressure wave is reflected at the surface of a body of greater density. Therefore, longlasting static overpressures of air would be expected to be of less importance than corresponding pressure waves in a surrounding medium of higher density.

Anesthetic level and underwater explosion damage. The great susceptibility to underwater blast damage of the animals at deep levels of anesthesia was somewhat surprising. In these animals with poor ventilation one might have expected a smaller gas/fluid interphase area and, consequently, smaller blast damage. The explanation may perhaps be sought in the fact that the ventilatory function of the deeply anesthetized rats was so depressed that the available respiratory area became insufficient after the blast, in spite of a moderate lung damage. This is based upon the observations shown in Fig. 5 where increasing anesthesia was followed by a larger increase of the lethality than of the lung damage.

References

Barcroft, J. *Sectional et el. kellers.* Report upon investigations of the standard of protection afforded. H. M. Stationery office. 1939.

Bein, A.-H. Explosions in water. A survey. *J. roy. nav. med. Serv.* 1954. 9. 191—200.

Celander, H., C. J. Clemedson, U. A. Ericsson and H. I. Hultman. A study on the relation between the duration of shock wa and the severity of the blast injury produced by it. *Act. physiol. sc. d.* 1955. 33. 14—18.

Clemedson, C. J. An experimental study on air blast injuries. *A. physiol. scand.* 1919. 18. suppl. 61.

Clemedson, C. J. Correlation between respiratory phase and extent of lung damage in air blast injury. *J. ppl. Physiol.* 1954. 7. 38—42.

Clemedson, C. J. and C. O. Carlson. Mechanical response of different parts of a living body to high explosion shock wave impact. *Amer. J. Physiol.* 1955. 181. 471—476.

Clemedson, C. J. and H. I. Hultman. Air embolism and the cause of death in blast injury. *Military S. geo.* 1953. 114. 424—437.

Clemedson, C. J., H. Hultman and B. Grönberg. Respiration and pulmonary gas exchange in blast injury. *J. ppl. Physiol.* 1953. 6. 213—220.

Krohn, P. L., D. Whitteridge and S. Zuckerman. Physiological effects of blast. *Lancet* 194. I. 252.

Wakeley, C. P. G. Effect of underwater explosions on the human body. *Lancet* 1915. I. 715—718.

Zuckerman, S. Experimental study of blast injuries to the lungs. *Lancet* 1940. II. 219—224.

Zuckerman, S. The problem of blast injuries. *Proc. roy. Soc. Med.* 1941. 34. 171—188.

Acta physiol. scand. 1968. 72. 15—24

From the Institute of Anatomy and Histology the Department of Obstetrics and Gynecology at the General Hospital of Malmö, University of Lund, and the Research Laboratories, Draco AB, Lund, Sweden

Histochemical and Pharmacological Studies on Amine Mechanisms in the Umbilical Cord, Umbilical Vein and Ductus Venosus of the Human Fetus

By

B EHINGER, G GENNSER, CH. OWMAN H. PERSSON and N-O SJÖBERG

Received 10 April 1967

Abstract

EHINGER, B., G GENNSER, CH. OWMAN, H. PERSSON and N-O SJÖBERG *Histochemical and pharmacological studies on amine mechanisms in the umbilical cord umbilical vein and ductus venosus of the human fetus* Acta physiol. scand. 1968. 72 15—24

The response in vitro of the umbilical vein and the ductus venosus to certain pharmacologically active drugs has been correlated with the adrenergic and cholinergic innervation of this venous system in human fetuses of 20—24 weeks gestational age. No histochemically demonstrable adrenergic or cholinergic nerves occurred in the umbilical cord. In the intra-abdominal part of the umbilical vein an increasing amount of adrenergic nerves were present in direction towards the ductus venosus. A distinct accumulation of adrenergic nerves was observed at the origin of the ductus venosus, concomitant with an increased thickness of the smooth muscle wall of the vessel. Few if any acetylcholinesterase-containing fibres could be demonstrated in relation to the umbilical vein or ductus venosus. Noradrenaline, acetylcholine, and 5-hydroxytryptamine produced contractile response in the intra-abdominal portion of the umbilical vein as well as in the first part of the ductus venosus. The noradrenaline response could be reproduced by tyramine and abolished by phenoxybenzamine, which potentiated the contraction induced by 5-hydroxytryptamine. Atropine inhibited the effect of acetylcholine, but not that of noradrenaline. The presence of aminergic nerves is not indispensable for the existence of vascular smooth muscle receptors for the amines. The accumulation of adrenergic nerves and smooth muscle cells in the wall at the origin of the ductus venosus supports the theory of an adrenergic sphincter mechanism at this by-pass.

The system comprised of the umbilical vein — within the umbilical cord as well as intra-abdominally — and the ductus venosus has received special attention in studies on fetal circulation because it allows oxygenated blood to pass directly from the placenta to the fetal heart and brain, and because this venous system involves special hemodynamic problems concerned with the regulation of blood flow from mother to fetus.

It is *well* established that certain pharmacologically active drugs (*e.g* catecholamines, 5 hydroxytryptamine (5-HT) histamine) and other stimuli, such as increase in oxygen tension, or thermal irritation, will cause a contraction in the cord vessels (Schmitt 1922, Haselhorst 1929 Runge 1936 Euler 1938 Panigel 1959) However the possible involvment of the autonomic nervous system is only little understood (see ten Berge 1962)

Although the umbilical vein undergoes distinct structural changes, for example reduction in the muscular elements, when entering the abdominal cavity (Barclay *et al* 1944) it could be expected that also the intra-abdominal portion should be have functionally similar to that part running within the umbilical cord both portions constitute an anatomically distinct unit of an unbranched vessel cond cting blood from the placenta to the fetus. When reaching the liver the umbilical vein gives off several branches, an important part of the blood being directed by way of the ductus venosus into the inferior caval vein.

Based upon the finding of smooth muscle fibres around the beginning of the ductus venosus Amoroso and Barron in 1942 suggested the presence of a sphincter mechanism at the junction between the umbilical vein and the ductus venosus (Barclay *et al.* 1944) Using angiographic techniques on fetal or newly born individuals, Lind *et al* (1964) could demonstrate in this region a temporary filling defect that supports the assumption of a sphincter Administration of noradrenaline or acetylcholine to newborn lambs, 3—6 days of age or stimulation of the vagus nerves supposed to innervate the sphincter (Barron 1944) always restored the patency of the ductus which is normally closed at this age (Peltonen and Hirvonen 1965) An accumulation of adrenergic nerves, demonstrated histochemically by the method of Falck and Hillarp has recently been registered at the origin of the ductus venosus, lending further support to the theory of a sphincter mechanism at this bypass (Gennser *et al* 1967)

The aim of the present investigation ha been to correlate the adrenergic and cl olinergic innervation of the umbilical vein system with vasomotor changes induced by the administration of certain biogenic amines under *in vit* conditions.

Material and methods

The material consis ed of human tissues, and ncluded umbilical cords taken immediat ly *post partum* as w ll as from fetuses of 21 and 24 weeks of gestational age Further preparations of the intra bdominal portion of the umbilical ein and he ductus enosus w re removed from fetuses 20—23 week old The mid-term material was obtained at legal abortions performed by laparotomy The ord preparations were examined *tel* whereas the other scula areas to be studied were as soon as possible carefully dissected free from adjacent tissues nder dissection microscope

Histochemistry / *ad ne gic nerv* Pieces, about 5 mm in length, were taken from the umbilical cords of 9 newborns and 2 mid term fetuses at fou sites the root in the abdominal wall, 10 mm from th bdominal wall, the intermedia part and the insertion into the placenta. The following part from 8 fetuses were dissected out the umbilical immediately inside the abdominal wall, the free in ra-abdominal portion of the ein, the first ntrahepatic part of the in, the junction between the ein and the ductus enosus, the intermediate part of the ductus, nd the junction of the duc with the inferior l ein

The preparations were quenched to the temperature of liquid nitrogen, freeze-dried, and treated with formaldehyde gas from paraformaldehyde t + 80° C for 1 hr for the histochemical demonstration of certain monoamines (F lck 1962, Falck *et al.* 1962, Corrodi and Hillarp 1963 1964) After embedding in paraffin, the pieces were serially sectioned at 6 μ thickness and nalyzed in the fluorescence microscope. (For further technical details, see Falck and Owman 1965) Under the conditions used, the adrenergic transmitter which can be visualized in the entire length of the sympathetic neuron (/ Falck and Owman 1966) exhibits an intense green fluorescence The identity of the green fluorophore was further assessed by comparing its excitation and emission spectra in the ductus enosus specimens with those of the noradrenaline-containing adrenergic nerves of the rat's vas deferens (Björklund 1965) using Leitz microspectrograph.

Histochemistry of cholinergic nerves. The technique for demonstration of acetylcholinesterase (Koelle 1963) is t present the only reasonably specific method available for localizing cholinergic nerves. The method was applied t pieces from 2 full-term umbilical cords, and from the intra-abdominal portion of the umbilical ein as well as the junction between the ein and ductus venosus, from 4 mid-term fetuses. The preparations were sectioned in cryostat at 10 μ thickness, and incubated for 3—4 hrs (and, in some cases, even up to 21 hr to assure that all stainable fibres appeared) in acetylthiocholine in the presence of Mipafox (N,N -di iso-propyl-phosphorodiamide fluoride 4×10^{-4} M) or iso-OMPA (tetra-*iso*propylpyrophosphoramide 10 M) to demonstrate acetylcholinesterase (Holmstedt 1957)

Histological demonstratio of smooth muscl s. Any accumulation of smooth muscle cells at the junction between the umbilical ein and ductus enosus was demonstrated in formalin-fixed specimens from tw mid-term fetuses. The material was serially sectioned (10 μ thickness) in the longitudinal plane and stained in hematoxylin -van Gieson (Romeis 1948)

Functional in-vitro studies. Preparations from 8 fetuses were used in the pharmacological experiments. The preparations were kept in Krebs solution, aerated with gas mixture of 95 % O and 5 % CO_2, when dissecting away adjacent tissues from the vascular areas to be utilized. Cylinders, about 2 mm long, of the umbilical vein were cut from approximately the same location in the fetuses just beneath the entral margin of the liver nd then suspended between tw loops of 5/0 ligature silk (John Weiss Ltd., London) loosely tied through the lumen of the vessel. The first 2 mm part of the ductus enosus, precisely at the origin from the umbilical ein, was prepared in similar way A narrow brim of the umbilical vein surrounding the entrance of the ein into the ductus was included to ensure that the preparations contained the mass of smooth muscle accumulation at this part of the ductus wall (Barclay *et al.* 1944) (Fig 1) The ascular cylinders were kept at room temperature in Krebs solution and bubbled with the gas mixture for at least 1 hr before the experiments were started.

The investigations on the ascular reactivity to arious pharmacological stimuli were made at 37 C in bath of 100 ml or 200 ml olume consisting of Krebs solution bubbled with the gas mixture to maintain the pH within physiological limits. The circular contractions in the essels were measured with Statham FT 03 force displacement transducer and recorded on Grass model 5D Polygraph. The muscles were allowed to adapt themselves to the new bath temperature for at least 1 hr before the registration was started. Between 10 and 20 experiments were performed on each preparation, which was thoroughly washed with prewarmed gas-bubbled Krebs solution and allowed to rest for more than 10 minutes before the next drug

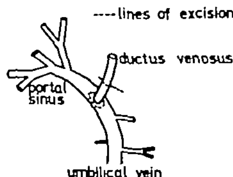


Fig. 1 The topographical relation of the hepatic part of the umbilical ein to the ductus enosus, showing that portion of the ductus (between the lines of excision) used in the *in vitro* studies.

test was started. The vessel wall was first given a load of about 100 mg. It usually adjusted itself to a tension of about 50 mg by subsequent lengthening. Only in one instance was it possible to maintain a tension of about 250 mg. The active substances tested were injected into the bath in volumes varying between 0.05 and 1.0 ml.

Results

The different parts of the afferent vein system from the placenta to the fetus showed a varying degree of adrenergic and cholinergic innervation. The excitation and emission spectra for the adrenergic nerves were identical with those of the noradrenaline-containing fibres in the rat vas deferens. Some regions appeared to lack both adrenergic and cholinergic nerves, other showed a clear accumulation of nerves, primarily of the adrenergic type. In all vascular segments investigated, it was possible to register a more or less definite response to the vasoactive drugs applied. The effects always manifested themselves as contractions; no clear dilatatory response was obtained.

Umbilical cord

Fluorescence microscopy of a large number of segments along the entire umbilical cord revealed the presence of adrenergic nerves only at that portion close to the umbilical ring. The green-fluorescent fibres having the appearance of smooth, preterminal axons were collected in a few bundles running in Wharton's jelly parallel to the vessels. The bundles showed no direct relation to the vessels and never split up into plexus formations, but ended blindly after running for only a short distance within the cord outside the abdominal wall. The distribution of adrenergic nerves was similar in the material from both mid term and full-term fetuses.

The umbilical cord (proximal part studied) contained no nerves with specific acetylcholinesterase.

A number of earlier investigations have established a marked reactivity of the umbilical cord vessels to certain vasoactive drugs; this particular section of the fetal circulation therefore received no further attention in the present pharmacological studies. Catecholamines, histamine and 5-HT all cause a marked constriction of the vessels, whereas the effect of acetylcholine is less pronounced, in some instances tending to produce a dilatatory response (Runge 1936, Euler 1938, Panigel 1959).

Intra-abdominal portion of umbilical vein

A varying number of adrenergic nerves were present in all preparations of the intra abdominal (but extrahepatic) part of the umbilical vein, except for that region of the vein located immediately inside the abdominal wall which usually contained no fluorescent fibres. The nerves never exhibited distinct varicosities typical of the terminal end of the axon. Although their fluorescence intensity was quite high, they did not run in plexus formations characterizing the terminal autonomic innervation apparatus. The high fluorescence intensity also of those adrenergic

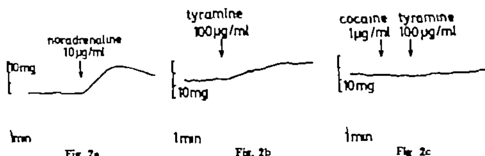


Fig 2. Umbilical vein Human fetus of 28 cm length.

nerves that are not immediately present in the terminal innervation apparatus is a characteristic feature of developing adrenergic neurons (Unpublished observations)

At least one thick bundle of fluorescent nerves accompanied the umbilical vein at some distance from its lumen. A moderate number of smaller bundles were usually also seen in the intra-abdominal tissue pieces, but they occurred more in relation to the small arteries and veins running in the mesenchyme outside the vein. Near the intrahepatic part of the umbilical vein, an increasing number of bundles was found, some splitting up into smaller branches consisting of only two to three fibres. These were often seen to radiate irregularly towards the wall of the vein.

No acetylcholinesterase-containing fibres occurred in immediate relation to the umbilical vein or in the mesenchyme outside the vessel.

The functional *in-vitro* experiments were always performed on the same segment, i.e. the final free part of the vein nearest to the liver Noradrenaline (0 1—10 µg/ml) produced a marked contraction in the preparation (Fig 2a) the strength of contraction being enhanced with increasing dose levels. In one preparation only no contractile response could be obtained even at doses up to 10 µg/ml. The contraction of the vein could be reproduced with tyramine (100 µg/ml) (Fig 2b) the effect being blocked by previous administration of cocaine (1 µg/ml) (Fig 2c) That preparation, which was unaffected by noradrenaline application, was also used for tests with 5-HT (0 02—1 µg/ml) This agent gave a contraction even more marked than that usually obtained by noradrenaline (Fig 3) the dose-response

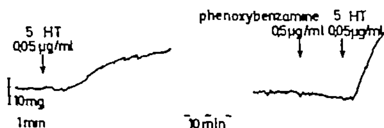


Fig. 3 Umbilical vein. Human fetus of 26 cm length.

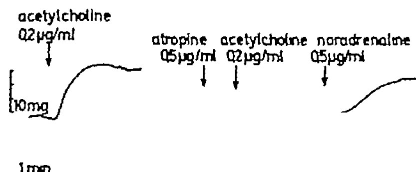


Fig. 4. Umbilical vein. Human fetus of 20 cm length.

pattern being very conspicuous. The 5-HT effect was strongly potentiated by phenoxybenzamine (0.5 µg/ml) (Fig. 3).

Acetylcholine (0.1—0.4 µg/ml) constantly caused a marked contraction (Fig. 4) that could be completely abolished by atropine (0.5 µg/ml). However atropine did not influence the subsequent response to noradrenaline (0.5 µg/ml) administration (Fig. 4).

Ductus venosus

The intrahepatic portion of the umbilical vein was surrounded by quite a large quantity of green-fluorescent nerve fibres, thus accentuating the increase in innervation already observed in the final free intra-abdominal part of the vein. A moderate number of coarse nerve bundles ran immediately outside the vessel, issuing smaller bundles which reached its wall. In tangential sections these small bundles were clearly seen to split up into a plexus formation superimposed upon the vein.

At the origin of the ductus venosus, the amount of nerves constituting the plexus formation increased suddenly (Fig. 5) concomitant with a thickening of the vascular wall and a reduction of the lumen calibre. In stained preparations this region was found to contain a distinct accumulation of smooth muscle fibres. It is notable that here the axons had a beaded appearance with varicosities interspersed irregularly along the fibres. Thus, the pattern suggests the presence of a terminal innervation apparatus at the junction of the umbilical vein with ductus venosus (Fig. 6). Within a short distance towards the caval vein, however, the amount of nerves in the ductus wall gradually decreased. Only few fluorescent nerves were present in the rest of the ductus and the lumen was wider (Fig. 5). The same scarcity of adrenergic nerve fibres was also registered at the junction with the caval vein.

The ductus venosus appeared to be devoid of acetylcholinesterase fibres, only small bundles being found to run at some distance from the vessel proper. Even incubations up to 21 hrs failed to reveal the presence of any further cholinergic nerves around the ductus (Fig.



Fig. 5. Human fetus 22 cm length. Demonstration of adrenergic nerves at the junction of the umbilical vei (uv) with ductus venosus (d) T the left, the hepatic part of the umbilical vein (uv) transforms ( ) I to ductus enosus (d) ha ing more narrow lumen. T the right the lumen again widens when the ductus (dv) continues in direction towards the I ferior caval vein. Only scattered fluorescent nerves I relation to the umbilical ein. At the junction with he ductus ( ) sudden increase is seen in the number of nerves running within the vessel wall. After short distance of rich innervation orresponding to rich supply of smooth muscle fibres in the wall, the amount of innervation in the ductus decreases, to remai slight in the rest of the ductus in direction towards the inferior caval vein. Fluorescence micrograph. 90 ×

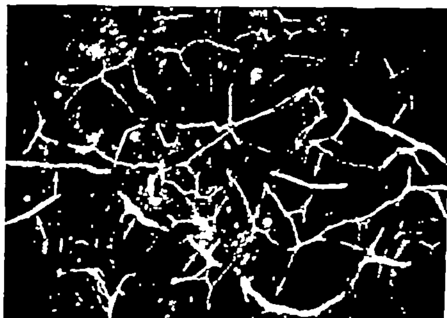


Fig 6. Human fetus 23 cm length Demonstration of adrenergic nerves in the first part of the ductus enosus, the junction with the umbilical ein. The essel has been cut open, flattened, and sectioned from the outside. Rich plexus formation in the vessel wall of green-fluorescent adrenergic fibres, running small bundles (arrow) or isolated. When isolated the fibres are seen t ha ricosed ppearance. Fluorescence micrograph. 100 ×



Fig 7 Huma fet 1 weeks old Demonstration of cholinergic nerves in first part of ductus venosus. *Lon tudinally and transversely sectioned nerv trunk (arrow) lie in the connective* tissue around the ssel, but no in direct ontact with t Incubation time *1 hr Mipafox* Phase contrast micrograph 200

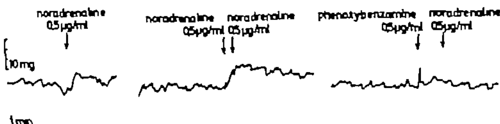


Fig. 8. Ductus venosus Human fetus 22 weeks old.

The part of the ductus venosus used in the pharmacological studies (seen in Fig 1) included the region of the supposed sphincter (Barclay *et al.* 1944) The response of this preparation to noradrenaline administration was not unequivocal. In two preparations, noradrenaline (0.5—1.0 µg/ml) caused a contraction that was reproducible and could be blocked by phenoxybenzamine (0.5 µg/ml) (Fig. 8) In ductus segments from six other fetuses, no response was obtained even with noradrenaline doses up to 10 µg/ml However when one of these failing preparations was used for experiments with 5-HT (0.1—0.5 µg/ml) or acetylcholine (0.5—5.0 µg/ml) a clear contractile response could be registered.

Discussion

Earlier investigations using conventional histological staining methods for demonstrating the arrangement of the peripheral innervation have indicated that certain areas of the afferent circulation to the fetus receive no or only few nerves (ten Berge 1962) The present histochemical results directly show that some regions are completely devoid of both adrenergic and cholinergic nerves (*e.g.* the major part of the umbilical cord) Other areas contain a small number of adrenergic but no cholinergic nerves (*e.g.* the free intra-abdominal segment of the umbilical vein) The arrangement of the nerves shows no direct relation to the vein proper Finally the junction of the umbilical vein with the ductus venosus exhibits a distinct accumulation of adrenergic nerves organized in a terminal innervation apparatus The few cholinergic nerves found are, on the other hand, present only at some distance from the vessel. It is notable that all vascular areas studied respond with definite contractions to the administration of noradrenaline acetylcholine and 5-HT Hence the absence of aminergic nerves to vessels does not immediately exclude the possibility of the existence of vascular smooth muscle receptors for the respective amines.

The accumulation of fluorescent nerves, and their characteristic arrangement around the origin of the ductus venosus which is provided with a conspicuous amount of smooth muscle fibres, yields further support for the theory of an adren-

ergic sphincter mechanism operating at this by pass. However a constant contractile response to noradrenaline administration could not be evoked under circumstances where 5-HT and acetylcholine could still elicit a positive response It therefore seems reasonable to exclude a technical failure as the cause of the non responsiveness to noradrenaline The inconstant vasomotor response to noradrenaline might rather be due to an uneven distribution of adrenergic receptors in the vascular system (Lemtis 1955) and or a different degree of maturity in e.g the enzyme mechanisms constituting the receptor (Ariens 1966)

The distribution of adrenergic nerves in the most proximal part of the umbilical cord indicates that they do not participate in the innervation of the cord vessels, but rather constitute aberrant fibres from the abdominal wall. The nerves, at least, could not be traced back to the intra-abdominal part of the umbilical vein.

Despite the scarcity of adrenergic nerves in the free intra abdominal segment of the umbilical vein, enough adrenergic transmitter is apparently available to provoke a contractile response Tyramine is well known to exert its sympathomimetic action through an uptake into the nerve followed by displacement of the adrenergic transmitter (Burn and Rand 1958) In the present result, tyramine produced principally the same effects as noradrenaline This effect could be abolished by cocaine which apparently blocked the uptake of tyramine into the nerves (Hertting 1965)

In a number of muscle preparations, acetylcholine has been shown to liberate noradrenaline from adrenergic nerve terminals (cf Burn and Rand 1965 Ferry 1966) In the umbilical vein, this does not seem to be the case since atropinization completely abolished all muscular response to acetylcholine (but not to noradrenaline) In other organs, direct peripheral interactions between adrenergic and cholinergic fibres have been demonstrated by Chiang and Leaders (1965) and by nger et al (1967) On the basis of the present experiments, it seems probable nd the receptors in ol ed in such an interaction are the ones responsible for the liberation of noradrenaline by acetylcholine since in the umbilical vein, where only adrenergic fibres occur acetylcholine did not seem to liberate noradrenaline It is remarkable that the absence of cholinergic nerves in immediate relation to both the umbilical vein and ductus enosus does not exclude the possibility of a marked contractile response to acetylcholine administration. This indicates that also circulating non-neural acetylcholine may ha e a physiologically significant action in the intact fetus.

Both noradrenaline and 5-HT produced a contraction in the umbilical ein as well as in the ductu enosus As recorded in the umbilical vein the noradrenaline response could be abolished by phenoxybenzamine (dibenzyline) which is a powerful blocking agent of the adrenergic receptors. It ha been suggested (Offermeier 1966) that 5-HT ma ha e an action on α-receptors, directly or indirectly In the present studies the ontractile action of 5-HT was, instead potentiated by phenoxybenzamine This indicates that the 5-HT receptor in the umbilical vein is not identical with the adrenergic α-receptor The increase of the contractile response to 5-HT registered after phenoxybenzamine i perhaps, at least partly due to a cocaine-like

effect (Hertting 1965) inhibiting a possible uptake of 5-HT into the adrenergic nerves (Andén *et al.* 1964 Bertler *et al.* 1964) thereby increasing the 5-HT concentration at the receptor site.

Supported by grants from the Association for the Aid of Crippled Children, New York, Magnus Bergwall Foundation, and the Swedish National Association against Heart and Chest Diseases.

References

ANDÉN, N. E., H. CORRODI, M. ETTLES, E. GUSTAFSSON and H. PERSSON, Selective uptake of some catecholamines by the isolated heart and its inhibition by cocaine and phenoxybenzamine. *Act pharmacol.* (Kbh.) 1964. 21. 247—259.

ARIËNS, E. J. Receptor theory and structure-action relationships. *In: Advanc. in Drug Res.* Ed. by Harper N. J. and A. B. Simmonds. Academic Press London and New York, 1966. 3. 235—285.

BARCLAY A. E., K. J. FRANKLIN and M. M. L. PRICHARD, *The Foetal Circulation.* Blackwell. Oxford. 1944. 1—275.

BARRON, D. H., The changes in the fetal circulation at birth. *Physiol. Rev.* 1944. 24. 277—295.

BREMER, B. S. von Nervenelemente in der Plazenta und Nabelschnur. *Geburtsh. Frauenheilk.* 1962. 22. 1228—1233.

BERTLER, A., B. FALCK and CH. OWMAN, Studies on 5-hydroxytryptamine stores in pineal gland of rat. *Act. physiol. scand.* 1964. 63. Suppl. 239. 1—18.

BURN, J. H. and M. J. RAND, The action of sympathomimetic amines in animals treated with reserpine. *J. Physiol.* (Lond.) 1958. *144.* 314—336.

BURN J. H. and M. J. RAND, Acetylcholine in adrenergic transmission. *Ann. Rev. Pharmacol.* 1965. 5. 163—182.

CHIANG, T. S. and F. E. LEADERS, Mechanism for nicotine and DMPP on the isolated rat tria-vagus nerve preparation. *J. Pharmacol. exp. Ther.* 1965. *149.* 225—232.

CORRODI, H. and N.-A. HILLARP. Fluorescenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 1. Identifizierung der fluoreszierenden Produkte aus Modellversuchen mit 6,7-Dimethoxyisochinolinderivaten und Formaldehyd. *Helv. chim. Acta.* 1963. 46. 2425—2430.

CORRODI, H. and N. A. HILLARP. Fluorescenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 2. Identifizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd. *Helv. chim. Acta.* 1964. 47. 911—918.

EHINGER, B., B. FALCK and H. PERSSON, To be publ. 1967.

EULER, U. S. v. Action of adrenaline, acetylcholine and other substances on nerve-free vessels (human placenta). *J. Physiol.* (Lond.) 1938. 93. 129—143.

FALCK, B. Observations on the possibilities of the cellular localization of monoamines by fluorescence method. *Acta physiol. scand.* 1962. 56. Suppl. 197. 1—25.

FALCK, B., N.-A. HILLARP, G. THIEME and A. TORP. Fluorescence of catechol amines and related compounds condensed with formaldehyde. *J. Histochem. Cytochem.* 1962. *10.* 348—354.

FALCK, B. and CH. OWMAN, A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta Univ. Lund.* *II.* 1965. 7. 1—23.

FALCK, B. and CH. OWMAN, Histochemistry of monoaminergic mechanisms in peripheral neurons. *In: Mechanisms of Release of Biogenic Amines.* Ed. by Euler U. S. v., S. Rosell and B. Uvnäs. Pergamon Press, Oxford. 1966. 59—72.

FERRY, C. B. Cholinergic link hypothesis in adrenergic neuroeffector transmission. *Physiol. Rev.* 1966. *46.* 420—456.

GENNSER, G., CH. OWMAN and N.-O. SJÖBERG, Histochemical evidence of an aminergic sphincter mechanism in the ductus venosus of the human foetus. *In: Proc. dings of the Symposium on Le Foetus Intrauterine Imminent.* Excerpta Medica Foundation, Amsterdam 1967. In press.

HERTTING, G. Effect of drugs and sympathetic denervation on noradrenaline uptake and binding in animal tissue. *In: Pharmacology of Cholinergic and Adrenergic Transmission.* Ed. by Koelle G. B., W. W. Douglas and A. Carlsson. Pergamon Press, Oxford. 1965. 277—288.

HOLMSTEDT, B. A modification of the thiocholine method for the determination of cholinesterase. II. Histochemical application. *Act. physiol. scand.* 1957. *40.* 331—337.

ergic sphincter mechanism operating at this by pass. However a constant contractile response to noradrenaline administration could not be evoked under circumstances where 5-HT and acetylcholine could still elicit a positive response. It therefore seems reasonable to exclude a technical failure as the cause of the non-responsiveness to noradrenaline. The inconstant vasomotor response to noradrenaline might rather be due to an uneven distribution of adrenergic receptors in the vascular system (Lemtis 1955) and/or a different degree of maturity in e g the enzyme mechanisms constituting the receptor (Ariëns 1966)

The distribution of adrenergic nerves in the most proximal part of the umbilical cord indicates that they do not participate in the innervation of the cord vessels, but rather constitute aberrant fibres from the abdominal wall The nerves, at least, could not be traced back to the intra-abdominal part of the umbilical vein.

Despite the scarcity of adrenergic nerves in the free *intra-abdominal segment of* the umbilical vein, enough adrenergic transmitter is apparently available to provoke a contractile response Tyramine is well known to exert its sympathomimetic action through an uptake into the nerve followed by displacement of the adrenergic transmitter (Burn and Rand 1958) In the present result, tyramine produced principally the same effects as noradrenaline This effect could be abolished by cocaine which apparently blocked the uptake of tyramine into the nerves (Hertting 1965)

In a number of muscle preparations, acetylcholine has been shown to liberate noradrenaline from adrenergic nerve terminals (*cf* Burn and Rand 1965 Ferry 1966) In the umbilical vein this does not seem to be the case since atropinization completely abolished all muscular response to acetylcholine (but not to noradrenaline) In other organs, direct peripheral interactions between adrenergic and cholinergic fibres have been demonstrated by Chiang and Leaders (1965) and by ...ger *et al.* (1967) On the basis of the present experiments it seems probable ...at the receptors involved in such an interaction are the ones responsible for the ...eration of noradrenaline by acetylcholine, since in the umbilical vein, where only adrenergic fibres occur acetylcholine did not seem to liberate noradrenaline It is remarkable that the absence of cholinergic nerves in immediate relation to both the umbilical vein and ductus venosus does not exclude the possibility of a marked contractile response to acetylcholine administration. This indicates that also circulating, non-neural acetylcholine may have a physiologically significant action in the intact fetu

Both noradrenaline and 5-HT produced a contraction in the umbilical vein as well as in the ductus venosus. As recorded in the umbilical vein, the noradrenaline response could be abolished by phenoxybenzamine (dibenzyline) which is a powerful blocking agent of the adrenergic-receptors It has been suggested (Offermeier 1966) that 5-HT may have an action on α-receptors, directly or indirectly In the present studies the contractile action of 5-HT was instead potentiated by phenoxybenzamine. This indicates that the 5-HT receptor in the umbilical vein is not identical with the adrenergic α-receptor The increase of the contractile response to 5-HT registered after phenoxybenzamine is perhaps, at least partly due to a cocaine-like

Acta physiol. scand. 1968. 72. 25—32

From the Laboratory for the Theory of Gymnastics, University of Copenhagen, Denmark

Thermoregulatory Responses to Arm Work Leg Work and Intermittent Leg Work

By

BODIL NIELSEN

Received 19 April 1967

Abstract

NIELSEN B *Thermoregulatory responses to arm work leg work and intermittent leg work.* Acta physiol. scand. 1968. 72 25—32.

The increase in esophageal temperature during continuous work on bicycle ergometer with the arms and with the legs was compared. Also the effect of intermittent leg work (30 sec rest/work intervals) was compared to that of continuous work. At the same rat of metabolic energy production the esophageal temperature in the steady stat was about the same independent of the different degree of anaerobicity and the different neuromuscular factors. It is therefore suggested that chemical factors libera ed in proportion to the aerobic processes is responsible for the tempera ture setting during work. The sweat rate nd the skin circulation expressed as conductance of be peripheral tissues varied in proportion to the total heat production independent of neuromuscular factors.

In a recent study of thermoregulation during uphill, level and downhill walking on a treadmill (B. Nielsen 1966) it was found that the increase of internal body tempera ture during exercise was independent of the total heat production, but was closely related to the metabolic energy production. This was considered a direct confirma tion of the theory of M. Nielsen (1938) that the increase in temperature during work is due to a setting of the body thermostat" at a higher level. It was suggested that a work factor of chemical nature produced by the oxydative processes in the muscles might be responsible for the setting of th work temperature level

Earlier studies on temperature regulation during work with different muscle groups i e arm work and leg work (Asmussen and M Nielsen 1947) showed a d fference in the rectal temperature in these two kinds of work, but results of B Nielsen and M. N elsen (1962) who measured also esophageal temperatures, indicated that the esophageal temperatures might be equal when th oxygen uptakes were the same. In the present experiments it was therefore planned to compare the esophageal temperature and the thermoregulatory reactions during exercise with the arm muscles and the leg muscles. Further the effect of intermittent work was compared

to that of continuous work with legs, in order to study the possible effects on the thermoregulating responses of the very high neuromuscular activity in the work phases.

Methods and procedure

The rectal temperature nd the esophageal temperature were measured thermoelectrically as described by B. Nielsen and M. Nielsen (1962). The rectal temperature was measured t four depths (27 22, 17 and 12 cm) and the esophageal temperature just bove the diaphragm. The readings are considered accurat to ± 0 025 C. The steady stat values used in the graphs are averages of 3 readings obtained between the 50th nd 60th minute of work.

The skin temperature was measured with skin thermocouple (B. Nielsen and M Nielsen 1965) which was placed against the skin at 15 sites. The verage skin temperature was calculated as the mean of the 15 measurements weighted according to the corresponding skin areas (Hardy and DuBois 1938). The error on the single skin temperature measurements is less tha 0,1 C.

The temperature of walls, ceiling and floor was measured with radiation receiver (B. Nielsen and M. Nielsen 1965) and the air temperature was read on 2 Hg thermometers hanging in front of and behind the subject.

The experiments were performed in climatic chamber Air and wall temperatures were kept t 27±0.3 C. An electric fan produced an air movement of bout 0,5—1 0 m/sec around the subject t chest height. The relative humidity was measured with aspiration psychrometer. The humidity was not controlled but ranged between 25 and 48 per cent RH and permitted an adequate evaporation of the sweat.

The work was done on Krogh bicycle ergometer suspended from balance (Krogh nd Trolle 1936) for continuous registration of the weight loss during uninterrupted work. (M. Nielsen 1938) The weight loss during work could be measured with an error of less than ± 5 g

For arm work the pedals were replaced by handles, and the subject sat in the ergometer hai with the legs resting on bar the foot of th chair Work ntensities of 252, 360, 450 and 540 kpm/min in the arm work nd 360 540, 720 nd 900 kpm/mi in the leg work experiments were used. Some of the leg work experiments were carried out as intermittent work using 30 sec work and 30 sec pause with 720 1080 1260 1440 and 1800 kpm/min, i.e. the same average work intensities as i the continuous leg work. The oxygen ptak in all types of work varied between 0 9 nd 2,5 l/min.

The respiratory gas exchange was determined by the Douglas-bag method nd air samples were nalysed duplicate on he Scholander pparatus (Scholander 1947)

The metabolic energy production was calculated from the oxygen uptake (using caloric value for oxygen of 4,9 kcal/l) The total heat production was found by subtracting from the energy uction the calorie equivalent of the external work.

Sweat production was obtained from the weight loss the steady stat (after 45—60 min work) by subtracting from the total weight loss the weight loss through the lungs due to eva ra ion nd respiratory gas exchange I the intermittent work experiments, where continuous eighing could not be btained, the weight loss was calculated from the weight lost between the 30 nd 60 mi of intermittent work. The heart rat was counted and change i the skin blood flow was estimated from alculations of conductance of peripheral tissues (Burton 1934 Winslow Herrington nd Gagge 1937 and Hardy 1937)

The subjects P S 21 years, 66 kg, 181 cm tall max aerobic capacity (estimated) bout 4 l as used i the ma part of the experiments But 24 expts w th arm and leg work were performed on subject *G N 23 years, 68 kg, 18? cm tall, max. aerobic capacity also bout 4 l, but less trained for* arm work

They arri ed the laboratory in he morning and sat 30 mi in the ergometer hair with the thermocouples in place before the start of the work experimen They worked for 60 min. I ernal temperatures were measured every 5 min, and skin emperature 4 times between the 30 and 60 min. Expired air was collected bout 40 and bout 50 min work nd the verage of the t determinations was used in the calculations.

In some experiments at the highes work ra sample of blood was draw from ein in the rm immedia ly af er work was stopped and the lactic acid conten was determined enzymatically (Biochemica Test Combina ion TC-B 15972, Boehringer nd Soehne GMBH, M nnheim)

Results

In Fig 1 the steady state level of esophageal temperature and in Fig 2 mean rectal temperature is plotted against the corresponding values of metabolic energy produc

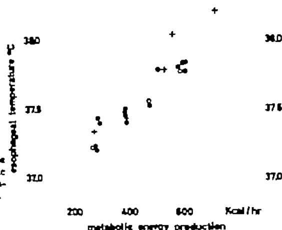


Fig. 1 Esophageal temperature in the steady state of work in relation to the rate of metabolic energy production. Environmental temperature 27,5 C. Subject PS.
○ arm work
● leg work
+ intermittent leg work

tion. Values for esophageal temperature during arm work and leg work fall on almost identical lines with only a slight tendency for lower values at the lowest arm work intensities. The intermittent leg work values plotted against average metabolic rate fall on the same line as the values from the continuous work experiments. The rectal temperature however is somewhat higher during leg work than in arm work. In the second, for arm work less trained subject, the esophageal temperature also during arm work was slightly lower (0,20—0,25 C) than that during leg work at the same rate of metabolic energy production.

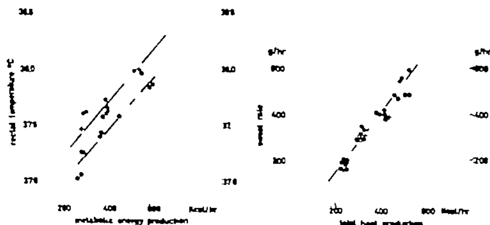
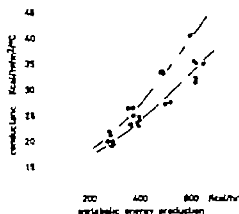


Fig. 2. Rectal temperature in the steady state of work in relation the rate of metabolic energy production. Environmental temperature 27.5 C. Subject PS.

Fig. 3. Sweat rate in the steady state of work in relation to the rate of total heat production. Environmental temperature 27.5 C. Subject PS.
○ arm work
● leg work
+ intermittent leg work



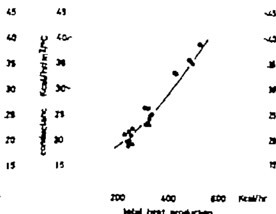


Fig 4 Conductance in relation to the rat of metabolic energy production. Environmental temperature 27.5 C. Subject PS.

Fig. 5. Conductance in relation to the rate of total heat production. Environmental temperature 27.5 C. Subject PS.
O arm work
● leg work

The relationship between the sweat rate and the rate of total heat production is illustrated in Fig 3 The correlation between sweat rate and total heat production is the same for arm work, leg work and intermittent leg work. Note that the sweat rate at the highest load of intermittent work is relatively low This is due to the extra high pulmonary water loss. The total water loss through lungs plus skin is similar to that obtained during continuous work.

The changes in skin circulation can be expressed as changes in conductance of the heral tissues. In Fig 4 and 5 this index of peripheral circulation is shown in ation to the metabolic energy production and the total heat production during arm work and leg work. The relationship between conductance and total heat production is the same for arm work and leg work. However when compared at the same metabolic energy production conductance is somewhat higher during arm work.

In Table I the values of mean skin temperature in the steady state of arm work and leg work and the oxygen uptake during arm and leg work is shown. Mean skin temperature was the same during arm work and leg work, and it was independent of the work intensity

The lactic acid content in the venous blood after 60 minutes of work was determined at the two highest work intensities with the arm and with the legs. After leg work the level of lactate was not increased above the normal resting level 7 4 mg while a small elevation was found after arm work, 23 7 mg . Further both heart rate and pulmonary ventilation increased more dur g arm work than during leg work for the same increase in metabolic energy production. (About 16 per cent higher HR and 20 per cent higher $\dot{V}_E$)

TABLE I. Average values of mean skin temperatures in the steady state of work and environmental temperatures at different work intensities during arm work and leg work. Subject PS

Subject	Number of expts.	Work rate kpm/min	$\dot{V}_{O_2}$	Mean skin temperature °C		Environm. temp.
PS				average	range	
arm work		252	0.95	31.3	(31.0—31.7)	27.35
	4	360	1.25	31.3	(30.7—31.5)	27.40
	4	450	1.62	31.4	(31.2—31.6)	27.45
	4	540	2.00	31.1	(30.2—31.6)	27.45
leg work	4	360	1.01	31.2	(30.6—31.6)	27.40
	4	540	1.36	30.7	(30.3—30.9)	27.30
	4	720	1.71	30.8	(30.1—31.7)	27.45
	5	900	2.10	31.1	(30.6—31.6)	27.40

Discussion

In the results presented above internal temperatures, sweat rate and skin circulation (expressed as conductance) have been compared in the steady state of different types of work i.e. intermittent work with the legs, continuous work with the legs and continuous work with the arms.

At the same rate of metabolic energy production (oxygen uptake) the neuromuscular conditions in the three situations are very different. During intermittent leg work the work load in the work phase was the double of that during continuous leg work. Therefore the mechanical tensions produced in the muscles, and the traffic of afferent and efferent nerve impulses in the work phase must have been much higher as compared to continuous work. During work with the arms a much smaller muscle group delivered the same energy output i.e. the strain on the single elements in the arm muscles must have been greater than in the leg muscles for the same rate of energy production, and the local temperature must have been higher. Also the local chemical factors must have been different judged from the lactic acid content in the mixed venous blood (see p. 28). In spite of these very different conditions both local and in the central nervous system, the increase in the esophageal temperature which is considered a good index of aortic blood temperature (Cooper and Kenyon 1957) is almost equal for the same metabolic rate in arm work, leg work and intermittent leg work (Fig. 1). In the second subject, who was less trained for arm work, the esophageal temperature was slightly (0,20—0,25 °C) lower during arm work.

In contrast to the findings regarding the esophageal temperature, the heart rate and pulmonary ventilation in both subjects was higher during arm work than during leg work. Also during intermittent leg work the average values (rest+work) of heart rate and pulmonary ventilation was higher than the values for continuous leg work for the same metabolic energy production (oxygen uptake).

In other words the level of esophageal temperature seems to be increased in proportion to the actual oxydative activity independent of the relative strain on the muscle group involved. The level of rectal temperature for the same metabolic energy production is higher (0,25—0 40 C) for leg work than for arm work in both subjects. This is probably due to a local heating of the rectum caused by the return of warm venous blood from the working muscles as pointed out by B Nielsen and M Nielsen (1962)

Recently Feldberg and Myers (1964) proposed a new theory of temperature regulation According to this concept the temperature regulation is mediated through the release of certain amines serotonine (5-HT) adrenaline and noradrenaline in the hypothalamus. They suppose that these amines may be continuously released and that the core temperature may be the outcome of the balance between the release of 5-HT and of the catecholamines. Further they suggest that the action of pyrogens and antipyretics may be explained as an interaction with this chemical balance.

Although studies on the actions of these amines in different species are not fully conclusive due to interspecific differences and to the reverse effect of large and small doses of the same substances (*cf* review by Cooper 1966) it seems that the balance between these particular amines do affect the body temperature level in a large number of animals.

Tentatively this theory may be applied to the problem in the present study The setting of body temperature during work in accordance with the metabolic rate (M Nielsen 1938 Robinson 1949 Wyndham *et al* 1952 Lind 1963 a and b B. Nielsen and M Nielsen 1962) In the light of the study by B Nielsen (1966) and the present data this setting may best be explained as being caused by some chemical agent liberated by or in proportion to the aerobic processes in the muscles. The action of such agents could take place peripherally via chemoreceptors or centrally The effect could be similar to the action atributed to a pyrogen by Feldberg and Myers (1964) i.e. a change in the normal balance between the catecholamines.

Considering the heat dissipating mechanisms the results presented in Fig 3 4 and 5 show that the sweat rate and the skin circulation hold the same correlation to ***total heat production*** for arm work, continuous leg work and intermittent leg work. This is in agreement with earlier findings, (B Nielsen and M Nielsen 1965 b B Nielsen 1966) In other words it seems unlikely that neuromuscular reflexes take part in the regulation of sweating during work (Beaumont and Bullard 1963 1966 Saltin and Hermansen 1966) Neither mechanoreceptors or cortical irradiation of motor nerve activity seems to be of importance for the sweat rate and the skin circulation in ***the steady state of work***

Robinson and co-workers (1965) proposed thermoreceptors in the muscles or in veins draining the working muscles as a more likely source of information to the thermoregulatory center than mechanoreceptors.

The local muscle temperature in the arms during arm work must have been higher than that in the legs during leg work. The same amount of heat produced in a small

muscle mass (the arms) and in a large muscle mass (the legs) could perhaps affect local thermoreceptors in such a way that the thermoregularoy center obtained information relative to the total heat production.

Another possible explanation for the correlation of sweat rate to the rate of heat production in the steady state is that given by Bazett (1951). That thermal receptors placed in different depths in the skin could provide information on the rate of heat flow through the skin. Or as Kerslake (1955) and Blockley (1966) propose that one set of receptors placed at a certain depth in the skin would be stimulated proportional to the rate of heat flow through the skin.

Summing up it can be stated, that in contrast with the respiratory and circulatory responses to work the thermoregulatory reactions do not seem to be influenced by the neuromuscular events during exercise.

References

ASMUSSEN, E. and M. NIELSEN: The regulation of the body temperature during work performed with the arms and with the legs. *Acta physiol. scand.* 1947, *14*, 373—382.

BAZETT, H. C., Theory of the reflex controls to explain regulation of body temperature at rest and during exercise. *J. appl. Physiol.* 1951, *4*, 245—262.

BEAUMONT, W. and R. W. BULLARD, Sweating, its rapid response to muscular work. *Science* 1963, *141*, 643—646.

BEAUMONT, W. and R. W. BULLARD, Sweating: exercise stimulation during circulatory arrest. *Science* 1966, *152*, 1521—1523.

BLOCKLEY, W. V., Human sweat response to activity and environment in the compensable zone of thermal stress. *NASA Report.* 1965, CR 65260 *Scientific and technical information division*, NASA, Washington D.C. 1966.

BURTON, A. C., The application of the theory of heat flow to the study of energy metabolism. *J. Nutr.* 1934, *7*, 497—533.

COOPER, K. E., Temperature regulation and the hypothalamus. *Brit. Med. Bull.* 1966, *22*, 238—242.

COOPER, K. E. and J. R. KENYON, A comparison of temperatures measured in the rectum, oesophagus and on the surface of the aorta during hypothermia in man. *Brit. J. Surg.* 1957, *44*, 616—619.

FELDBERG, W. and R. D. MYERS, Effects on temperature of amines injected into the cerebral ventricles. A new concept of temperature regulation. *J. Physiol. (Lond.)* 1964, *173*, 226—237.

HARDY, J. D., The physical laws of heat loss from the human body. *Proc. nat. Acad. Sci. (Wash.)* 1937, *23*, 631—637.

HARDY, J. D. and E. F. DUBOIS, The technic of measuring radiation and convection. *J. Nutr.* 1938, *15*, 461—475.

KERSLAKE, D. MCK., Factors concerned in the regulation of sweat production in man. *J. Physiol. (Lond.)* 1955, *127*, 280—296.

KROGH, A. and C. TROLLE, A balance for determination of insensible perspiration in man and its use. *Skand. Arch. Physiol.* 1936, *73*, 159—162.

LIND, A. R., A physiological criterion for setting thermal environmental limits for every day work. *J. appl. Physiol.* 1963a, *18*, 51—56.

LIND, A. R., Physiological effects of continuous or intermittent work in the heat. *J. appl. Physiol.* 1963b, *18*, 57—60.

NIELSEN, M., Die Regulation der Körpertemperatur bei Muskelarbeit. *Skand. Arch. Physiol.* 1938, *79*, 193—230.

NIELSEN, B. and M. NIELSEN, Body temperature during work at different environmental temperatures. *Acta physiol. scand.* 1962, *56*, 120—129.

NIELSEN, B. and M. NIELSEN, On the regulation of sweat secretion in exercise. *Acta physiol. scand.* 1965a, *64*, 314—322.

NIELSEN, B. and M. NIELSEN, Influence of passive and active heating on the temperature regulation of man. *Acta physiol. scand.* 1965b, *64*, 323—331.

NIELSEN, B., Regulation of body temperature and heat dissipation at different levels of energy- and heat production in man. *Acta physiol. scand.* 1966, *68*, 215—227.

Robinson, S., *Physiological adjustments to heat.* In: Physiology of heat regulation and the science of clothing. Chap. 5, ed. L. H. Newburg. (Saunders, Philad.) 1949.

Robinson, S., F. R. Meyer, J. L. Newton, C. H. Ts'Ao and L. O. Holgersen, Relations between sweating, cutaneous blood flow and body temperature in work. *J. appl. Physiol.* 1965. *20.* 575—582.

Saltin, B. and L. Hermansen, Esophageal, rectal and muscle temperature during exercise. *J. appl. Physiol.* 1966. *21.* 1757—1762.

Scholander, P. F., Analyser for accurate estimation of respiratory gases in one-half cubic centimeter samples. *J. biol. Chem.* 1947. *167.* 235—250.

Winslow, C.-E. A., L. P. Herrington and A. P. Gagge, Physiological reactions of the human body to varying environmental temperatures. *Amer. J. Physiol.* 1937. *120.* 1—22.

Wyndham, C. H., W. v. d. Bouwer, M. G. Devine and H. E. Paterson, Physiological responses of African laborers at various saturated air temperatures, wind velocities and rates of energy expenditure. *J. appl. Physiol.* 1952. *5.* 290—298.

Acta physiol. scand. 1968. 72. 33—44

From the Institute of Physiology, University of Oslo, Norway

Conditions Governing the Pressor Response to Ventilation Hypoxia in Isolated Perfused Rat Lungs

By

ANTON HAUGE

Received 24 April 1967

Abstract

HAUGE, A. *Conditions governing the pressor response to ventilation hypoxia in isolated perfused rat lungs.* Acta physiol. scand. 1968. *72.* 33—44.

An isolated perfused and ventilated lung preparation from the rat is described. After perfusion for 16—30 min the preparation responded with a reversible increase in pulmonary vascular resistance to short (3—4 min) periods of ventilation hypoxia (gas mixtures containing 2—7 % O_2). This pressor response to hypoxia reached a maximum 45 to 140 min after start of the perfusion. The response then declined and disappeared, whereas the preparation did still respond well to vasoactive drugs. This vulnerability of the pressor response to hypoxia is in agreement with the findings in isolated lung preparations from other species. In this preparation the disappearance of the response could be shown to be due to changes within the lungs themselves, and not to changes in the perfusate blood. None, or very slight, pressor responses to ventilation hypoxia could be elicited in preparations where platelet-poor plasma was used as a perfusate. If, however, the formed elements of the blood were added to the plasma perfusate, good responses to ventilation hypoxia appeared, provided that the addition was done during the first hour of perfusion.

Observations on the increase in pulmonary arterial pressure caused by asphyxia dates back to the turn of the century. The possibility that changes in alveolar gas tensions might have an effect on pulmonary vessels was also discussed by some of the early workers in this field (for review see: Fishman 1961, Daly and Hebb 1966). The more recent interests in the mechanism whereby alveolar hypoxia causes an increase in pulmonary vascular resistance arose from the important work of Euler and Liljestrand in 1946. They demonstrated in experiments on anesthetized cats that a low oxygen tension in the alveolar air induced a marked rise in pulmonary arterial pressure. They also suggested that the response was part of a mechanism involved in the distribution of pulmonary blood flow. Since then a number of investigators have examined further this response to acute alveolar hypoxia, the presence of which is well established in several animals and also in man (Fishman 1961, Daly and Hebb 1966).

A pressor response to acute alveolar hypoxia has also been found in isolated perfused lungs from various animal species such as the cat (Nisell 1948, Duke 1951), the

dog (Duke 1957 Daly and Wright 1957) and the rabbit (Waaler Hauge and Lunde 1966) indicating that at least part of the mechanism behind this effect is located within the lungs themselves. The response is, however not easy to maintain in isolated lung preparations, and in several investigations it has not been obtained at all (Aviado Ling and Schmidt 1957 Allison Daly and Waaler 1961) In isolated lung preparations which do give this pressor response, more severe alveolar hypoxia seems to be necessary for its elicitation than what is needed for its demonstration *in vivo* Aviado *et al* (1952) in one series of investigations found their dog lung preparations to be responsive to 5 to 10 oxygen in nitrogen for a period of only 30 min. Lloyd (1964) has reported that the pressor response to acute alveolar hypoxia in isolated blood perfused dog lung preparations disappears after about 60 min of perfusion. Also, on reading several of the papers of Nisell (1948, 1950) and Duke (1951 1957) and that of Duke and Killick (1952) on the pulmonary vascular effect of ventilation hypoxia in isolated lungs, one gets the impression that they have been dealing with a delicate and perishable response. Daly Ramsay and Waaler (1962) pointed out that the presence of such a response in isolated perfused dog lung lobes depended on the time interval between interruption of normal circulation and start of perfusion (the ischemic period) and upon the temperature of the blood perfusate

Isolated blood perfused lungs from *e g* rabbits can be maintained in a generally good state for at least 5 hrs of perfusion as judged from the lack of edema formation and from the reactivity of the vascular smooth muscle to vasoactive drugs (Hauge Lunde and Waaler 1966a) In a recent study Levey and Gast (1966) found that isolated blood perfused rat lungs metabolized glucose for at least 3 hrs of perfusion On this background the marked vulnerability of the pressor response to alveolar hypoxia is of particular interest Conditions governing the presence and disappearance f the response may provide clues to the understanding of its nature and its mode of ion in the vascular bed The present tudy was undertaken in order to analyze re closely parameters which might ccount for th vulnerability of the response hanges in the perfusate as well as changes within the lungs themselves were consider ed The preparation used was isolated perfused lungs from the rat To our knowledge the effects of al eola hypoxia on pulmonary ascular resistance has not previously been studied in this species.

Methods

Anesthesia. The animals from hich he lung prepara ions were to be taken, were nesthetized by a intraperi oneal jection of 3 4 mg 100 g body weigh of pentoba bitone (Nembutal® Abbott, diluted 1:5 with isotonic sal ne Blood donor nimal were nesthetized with ether

The lung preparation was taken from inbred lbino rats (local strain originally Wistar) of either sex weighing bet een 180 nd 280 g The hest of he anesthetized animal was opened during positiv pressure entilation T e hea , lungs nd he grea er essels were dissected free from djacent tissue nd loose ligatures were ied round the al essels Half ml of solution contai ing 100 I u. of pure powdered heparin Nov otoni saline was slowly injected int the left entricle whereafter the ca al ligatures ere igh ened nd he entila on stopped A curved tainless steel inflow cannula was inserted through he righ entricle to he pulmonary artery The outflow cannula wa inserted through the lef en ricle nt he left rium and f stened by tight lig ture around the heart entricles. One end of thi lig ure as used for suspension of the preparation (see

below) which was now removed from the chest. The preparation consisted of both lungs with the trachea, the heart and the pulmonary vessels. The time between the arrest of the animals own circulation and start of the perfusion was between 9 and 33 min, in most experiments around 15 min.

Perfusion. The preparation was hanging freely inside closed cylindrical perspex chamber surrounded by thermostated water-jacket. The preparation was suspended in string fastened around the heart-ventricles and the two perfusion cannulas. The temperature of the perfusate and that in the chamber were kept between 36 and 38°C. The pulmonary vascular bed was perfused with constant volume pulsatile inflow using piston driven pump with ball valves, described by Iversen and Steen (1966) The flow was adjusted from the outset, when inflation of the lungs was also carried out, so that the mean pulmonary arterial pressure was within the normal range reported for rats (mean 18.8 mm Hg (Smith and Bennet 1934)) The pulmonary arterial pressure was remarkably stable throughout each experiment. The flow rates obtained with whole blood usually corresponded the lower range of the normal cardiac output reported for rats (17—76 ml/min mean 47 ml/min (Spector 1956))

The blood reservoir consisted of an open, double walled thermostated glass container The pump lifted the perfusate from bottom outlet of this reservoir and pumped it through the arterial cannula into the preparation. The inflow pressure was measured continuously with Statham P23Gb *pressure transducer connected to Sanborn Model 320 DC amplifier recorder* via Sanborn Model 350-1100 B DC preamplifier From the outlet (left atrial) cannula the perfusat was drained into the blood reservoir For connections were used silicone rubber tubings. Left atrial pressure was kept constant throughout each individual experiment, at level between 3 and 6 cm of water

Ventilation. After one or more gentle inflations of the lungs, positive pressure ventilation was carried out with small Starling Ideal pump (C. F Palmer (London) Ltd.) using the ventilation overflow arrangement described by Konzett and Rössler (1940) Peak inspiratory pressure was kept at 10 *cm of water and expiratory pulmonary pressure was kept at 1.5—2.0* cm of water Ventilation overflow was recorded on kymograph with Palmer 4 ml piston recorder The ventilation frequency was 34/min.

The standard gas mixture used for ventilation was 6% CO_2 in air In order to create alveolar hypoxia two other gas mixtures were used 1) 7% O_2 6% CO_2 and 85 % N_2 and 2) 2% O_2 6% CO_2 and 90% N_2 (called the 7 and 2 oxygen gas mixtures, respectively) In few experiments hydrogen was substituted for nitrogen in gas mixture no. 2.

Perfusate Usually 3 donor rats were exsanguinated by heart puncture. To each 10 ml of blood was added 0.5 ml of heparin solution (100 I.U.) in saline. The volume of the perfusate varied from 17 to 25 ml. In experiments involving exchange of whole blood with plasma as perfusate 5 or 6 donor rats had to be exsanguinated. The perfusate blood was mixed and *kept in* closed glass container at 5 C until 5 min prior to the mounting of the lung preparation. Platelet-poor plasma was prepared by centrifuging whole blood at 5000 g for 20 min and pipetting off the supernatant plasma. Due to slow gradual release of platelets from the pulmonary vascular bed during the perfusion the number of circulating platelets was usually between 20,000 and 45,000 mm of blood after 1 hr of perfusion with this perfusate.

P_{O_2} of effluent perfusate was followed continuously with Beckman micro oxygen electrode and Physiological Gas Analyzer (Beckman) *Model 160, and recorded on Sanborn Model 320 DC* amplifier recorder

Hematocrit values were estimated using an International micro-capillary centrifuge model MB.

pH of the perfusate was measured with Metrohm Herisau pH Meter E 300 B. Glass electrode specification EA 120—3

Drug addition. Drug injections were carried out into the pulmonary arterial tubing Some drugs were added to the blood reservoir in order to obtain rapid mixing with the perfusate

List of drugs used. Adenosine *triphosphate (ATP) (Adenosine 5-triphosphate disodium salt* crystalline from Equine Muscle Sigma Chemical Company) Bradykinin (Synthetic Bradykinin, BRS 640, Sandoz A. G) Histamine (Histamine di-hydrochloride, Roussel-Uclaf Kallidin (Synthetic Kallidin, KL 698, Sandoz A. G)

Results

Each lung preparation was perfused for at least 3 hrs Perfusate blood pH during the first 30 min of perfusion varied between 7 38 and 7 48 A gradual fall in perfusate pH in the order of 0 04—0 06 units/hr was thereafter regularly seen

In 25 out of 25 blood-perfused preparations could marked increases in pulmonary vascular resistance (PVR) be obtained on ventilation with gas mixtures containing

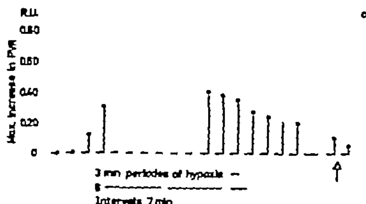


Fig. 1. *Development of pressor response to ventilation hypoxia in an isolated rat lung preparation.*
Standardized periods of ventilation with the 2 oxygen gas mixture repeated at regular (7 min) intervals. *Perfusion started 10 min prior t the first hypoxic period.* Blood flow through preparation 16 ml/min. | Maximal increase in pulmonary ascular resistance (PVR) during ventilation with 2° oxygen gas mixture | Maximal increase in PVR after the injection into the *arterial tubing of 200 µg of ATP. The arrow indicates the exchange of the old perfusate blood with freshly collected blood from two donor rats. R. U* Resistance units (pulmonary arterial pressure — left terial pressure/blood flow mmHg ml/min)

2 oxygen. The pressor response developed in a characteristic way when 3 or 4 min periods of al eolar hypoxia were applied at regular intervals from the outset of the perfusion. During the first 16—30 min no pressor response could be elicited. In the following period the response was gradually increasing in size until it became maximal between 45 and 140 min after start of perfusion. Shortly thereafter the response started to decline and finally it could no more be elicited. An experiment of this type is shown in Fig. 1.

During the whole of this response-cyclus, and also during the rest of the perfusion 2 hrs arterial injections of vasoconstrictor drugs, such as g bradykinin or y ladin in doses of 100 or 200 µg caused a reproducible and marked transient rease in PVR.

The mean ischemic period of the 23 blood-perfused preparations in which the hypoxic pressor esponse could be evoked was 17 min with a variation from 9 to 33 min. No correlation was found between the length of the ischemic period within these limits and durat on and intensity of responsiveness. The two blood perfused preparations in which no responses were obtained had ischaemic periods of 13 and 1? min respectively.

In order to determin whether the appearance of the first pressor response to alveolar hypoxia was determined by the elapsed time of perfusion alone or by a combined effect of perfusion time and non-effective hypoxic periods, the start of the first period of hypoxic entilation was deliberately delayed until 45 min after the start of perfusion. Further hypoxia tests were carried out at 30 min intervals. An experiment of this type is illustrated in Fig. 2. The pressor response to hypoxia was here present on the first trial but then only in a few of the subsequent tests. These findings were confirmed in two similar experiments. There thus seemed to



Fig 2. Pressor responses in an isolated rat lung preparation to periods of ventilation hypoxia which were applied late in perfusion, and at long intervals.
Four periods of ventilation with 2 % oxygen gas mixture were applied at intervals of 30 min, the first one 45 min after start of perfusion. Time after start of perfusion is given below. Signals indicat beginning and end of hypoxia periods. P.A p. Pulmonary arterial pressure. Pv Oxygen tension in venous (effluent) blood. VOF Ventilation overflow which equals effecti stroke volume of respiration pump (here about 8 ml) minus tidal volume. Blood flow through preparation 10 ml/min.

be a tendency towards decline in responsiveness to hypoxia with time of perfusion, whether frequent or less frequent hypoxia periods were pplied.

The size of the pressor responses to hypoxia was related to the degree of alveolar hypoxia and to the duration of the hypoxic period. Thus ventilation with gas mixtures containing 7% of oxygen was without effect at an early stage of the perfusion, when a moderate pressor response to gas containing 2 % of oxygen had been established (Fig 3) The same was seen late in the perfusion, when the responses to hypoxia had started to decline (Fig 4) However at the time when maximal responses to gas mixtures containing 2 % oxygen were obtained, ventilation with gas mixtures containing 7% oxygen also gave marked, although somewhat smaller pressor responses (Fig 3)

This difference in responses seemed to be due not only to the difference in oxygen content in the two gas mixtures, but also to the difference in the rate of fall in al eolar P_O Thus in 2 expts. the pressor response to gas mixtures containing 2 oxygen tarted at a level of effluent blood P_O (P_{V_O}) similar to, or slightly above, that of the lowest P_{V_O} value obtained during non-effecti e ventilation with gas mixture containing 7 oxygen.

Th effect upon the hypoxia-induced pressor response of a prolongation of the hypoxic period is demonstrated in Fig 1 A doubling of this period markedly increased the rise in PVR. A similar effect of a prolongation of the hypoxic period was seen at all stages during the reactive phase of the preparations. In the period f maximal reactivity ventilation for 1 min with gas mixture containing 2 of oxygen or for 3 min with a gas mixture containing 7 of oxygen would usually cause the



Fig. 3. *Pressor responses to two different degrees of ventilation hypoxia at an early stage of perfusion.* Beginning and end of periods with ventilation hypoxia are marked with arrows. Time after start of perfusion is given below. Note the slower development of the pressor response to the 7% oxygen gas mixture as compared with that to the 2% oxygen gas mixture. In the third ventilation test nitrogen was replaced by hydrogen in the low oxygen gas mixture. Note the marked bronchomotor activity in this test. Blood flow through preparation 16 ml/min. Abbreviations as in Fig. 2.

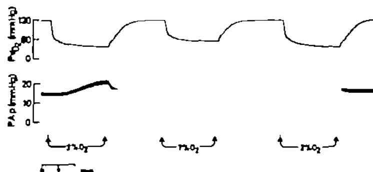


Fig. 4. *Pressor responses to two different degrees of ventilation hypoxia at a late stage of perfusion.* Perfusion started 120 min prior to the first period of hypoxia illustrated here. Abbreviations as in Fig. 2.

ry arterial pressure to rise to levels of from 30 to 50 mm Hg and there would be no apparent tendency for the pressure to level out.

During this period of high sensitivity the pressor response was seen to start within 10—15 sec after the entrance of hypoxic gas into the airways. With flow rates from 16 to 20 ml/min it takes 50—60 sec to recirculate the whole of the perfusate. Thus the pressor response started at least 30 sec before the P_{O_2} of the inflowing (arterial) blood could have started to decrease. This is in agreement with the findings of Duke (1951) and Lloyd (1964) that the pressor response was unrelated to arterial P_{O_2}. The rise in PVR on alveolar hypoxia was always readily reversible, the fall in pressure usually appearing within 10—20 sec after the first entrance of oxygen rich gas mixtures into the airways.

In order to separate possible effects of the high nitrogen content of the ventilation gas from effects of its low oxygen content, nitrogen was replaced by hydrogen in 4 expts. The oxygen content of the gas mixtures as well as the carbondioxide content was kept constant at 2 and 8 % respectively. Duke (1951) found no change in the responses in isolated dog lungs by replacing nitrogen with hydrogen, a finding

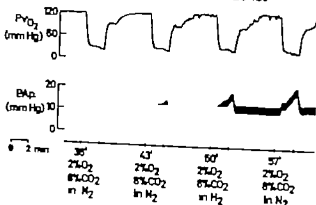


Fig. 5. *Effect on pressor response to ventilation hypoxia of replacing nitrogen in the hypoxic gas mixture with hydrogen.*

First four 2 min-tests with ventilation hypoxia in one experiment are shown. Time after start of perfusion and composition of gas mixtures are gi en below Blood flow through preparation 11 ml/min. Abbreviations as in Fig. 2. Exchange of one "inert gas with another did not influence the gradual steady increase in responsiveness of the preparation.

which was confirmed in this preparation (Fig 3 and 5) Ventilation with 90 hydrogen did, however induce more bronchomotor activity (Fig 3) than did ventilation with 90% nitrogen.

The disappearance of the hypoxic response in an otherwise vital and well-reacting preparation could be due to changes in the perfusate, to changes within the lung tissue itself, or to both. In an attempt to analyze this problem, the perfusate blood in a lung preparation, in which the hypoxic pressor effect had been nearly exhausted, was replaced by freshly collected, whole, autologous blood (Fig 1) No increase in the declining hypoxic pressor response was seen after the blood replacement. In another similar experiment, where the pressor effect of hypoxia had been completely exhausted, no reappearance of the response was seen after replacement of the blood perfusate. Nor could the pressor response be regained by increasing plasma glucose concentration by 100 mg/100 ml

In two other experiments the pressor response to hypoxia had been exhausted by repetitive 3 min tests with a gas mixture containing 2 % oxygen. Using the same blood perfusate, the lungs themsel es were now replaced with a freshly prepared new pair of lungs. The original blood perfusate was kept circulating also during the installation of preparation no 2. The "ischemic periods of the two pair of lungs were equal to within 1 1/2 min. One experiment of this type is shown in Fig 6 Only 4 out of 8 hypoxia tests are shown from the first lung preparation and 4 out of 9 from the last preparation. The second pair of lungs displayed a similar development of the pressor responses to hypoxia as did the first preparation. Initially there was a non-reactive period. Thereafter increasing responses were seen and finally the responses declined and disappeared. The exhaustion of the pressor response to hypoxia in preparations perfused with whole blood is thus apparently due to changes in the lungs themsel es.

In order to analyze further the role of the perfusate 5 expts were carried out in

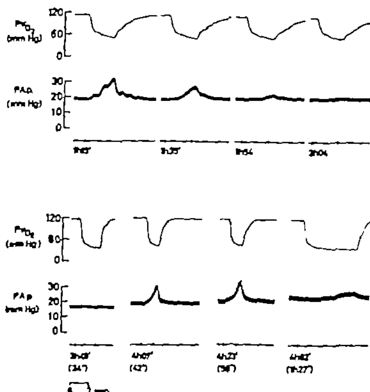


Fig. 6. *Pressor response to ventilation hypoxia in two isolated rat lung preparations, subsequently perfused with same blood perfusate*

Upper row pressor responses to 4 (of total of 8) periods of ventilation hypoxia i one lung preparation After the disappearance of the pressor response, the lungs were replaced with a new second preparation, whereas the same perfus blood was being used. The first 6 ml of effluent blood from preparation no 2 were discharged. Lower row pressor response t 4 (of total of 9) iods of entilation hypoxia in his second preparation. The 2 oxygen mixture used in all tests. after start of the first perfusion is given without parenthesis. Time after start of he second rfusion is gi en n parenthesis. Blood flow through 1st preparation 18 ml/min. Blood flow through d preparation 20 ml/mi Abbreviations as Fig 2

wh ch the lungs were perfused with platelet poor plasma Care was taken to wash out whole blood remaining in the vascular bed of the lungs as they were taken out from the animal Thus the first 8—10 ml of effluent perfusate were discarded No pressor responses to ventilation hypoxia developed in 3 of these preparations, whereas a few weak responses were obtained n the 2 other ones.

In 2 expts. plasma perfusion was tried subsequen to an initial period of whole blood perfusion. The blood perfusat was replaced by platelet poor plasma (see Methods) when the pressor response to hypoxia had been established An experiment of this type is illustrated in Fig 7 The changes from whole blood to plasma caused a decrease in the inflow pressure and a moderate decrease in tidal volume The pressor response to hypoxia was, however clearly present also with pla ma as perfusat It took 10 2—3 min periods of ventilat on hypoxia before the response was exhausted. At this point all the corpuscular element of the blood which had previously been removed by centrifugation (see Method) were added — in order to regain the

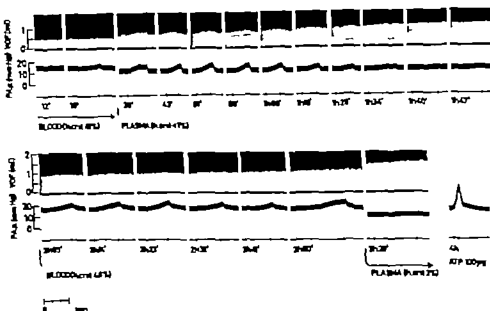


Fig. 7. *Effects of presence of blood corpuscles in perfusate on pressor response to ventilation hypoxia.*
Consecutive tests with 2—6 min periods of ventilation with the 2% oxygen gas mixture are shown. During first 2 tests whole blood was used as perfusate. During the following 10 tests plasma was used as perfusate. Then red blood cells and buffy coat were added to perfusate to normal hematocrit for subsequent 6 tests. A second exchange of blood with plasma carried out before last test. Beginning and end of periods with ventilation hypoxia are marked with small vertical bars on the time line. ATP 100 µg, was injected at the end of the experiment. Flow was kept constant at 18 ml/min. Abbreviations as in Fig. 2.

normal perfusate composition. During the perfusion with plasma these blood corpuscles had been kept in an air tight glass container at 5 C. The addition of the blood corpuscles to the perfusate had moderate immediate effects on the preparation with a small increase in tidal volume and a moderate increase in perfusate inflow pressure. More interesting was the fact that the pressor response to hypoxia was regained for a certain period of time. Before the response was again completely exhausted, plasma was once more introduced as perfusate. This time no further pressor response to hypoxia could be obtained with this perfusate, whereas the preparation did still respond with a marked vasoconstriction to 100 µg of arterially injected ATP.

In 3 perfusions platelet-poor plasma from a certain blood portion was initially used as perfusate and the removed corpuscular elements then added to normal hematocrit after a certain period of time. Good responses to hypoxia were obtained after addition of the blood corpuscles, provided that the addition took place within 60 min after start of perfusion.

The bronchial vascular bed was not perfused in this preparation. Nevertheless bronchoconstriction, as judged by the decrease in tidal volume, could be obtained in more than 2/3 of the preparations by the injection of kallidin, histamine or bradykinin into the pulmonary arterial tubing. Ventilation with the 2 oxygen gas

mixture also usually evoked a slight reduction in tidal volume synchronous with the pressor effect (Fig 7)

Discussion

In spite of many investigations and a large number of valuable observations accumulated from them, we do still not know which intrapulmonary mechanisms are involved in the vascular response to acute alveolar hypoxia.

This investigation shows that the pressor response to acute alveolar hypoxia is present also in isolated blood perfused lungs of the rat, and that the responsiveness of the preparation to hypoxia follows a characteristic pattern Only within a limited span of time could the pressor response to hypoxia be obtained whereas the preparation remained in a generally good state for a much longer period of time as judged by other criteria The responses to vasoactive drugs were thus unchanged at a time when responses to ventilation hypoxia could no more be evoked.

The decline and disappearance of the pressor response to hypoxia went on seemingly independent of the number of responses evoked Thus, a relatively large number of responses to 3 or 4 min periods of ventilation hypoxia could be obtained within the responsive phase This phase could not be extended by delaying the tests or by increasing the interval between each period of ventilation hypoxia. A quantitative approach to this problem is difficult however due to the variation in all over responsiveness to hypoxia from one preparation to the other This variation could in this study not be related to the length of the ischemic period within the given extremes 9—33 min) This does not necessarily disagree with the findings of Daly *et al* (1962 *that the pressor response to ventilation hypoxia could not be obtained* in dog lungs where the ischemic period had been longer than 35 min With ischemic periods of from 12 to 35 min they found that the pressor response to ventilation hypoxia could be obtained only at a perfusate temperature of 35 C or more Low temperature perfusions were not included in the present study

The experiments with change of perfusate and test organ show that the disappearance of the presso response in blood perfused lungs is due to changes within the lungs themsel es Freshly collected blood perfusate could not revive the response once it had been lost, whereas new lungs perfused with the old perfusate responded in the usual pattern

The effects of ariations in the composition of the perfusate upon the pressor response to vent lation hypoxia ha e been in estigated in isolated cat lungs by Duke and Killick 1952 who found that the pressor response could be evoked w th Ringer Locke solution or dextran as perfusate and with arious dilutions of blood with dextran. There was evidence hov ever that the vasoconstrictor effect of N wa somewhat reduced w th these perfusates.

Lloyd reports that no pressor response could be obtained in solated dog lungs perfused with a buffered dextran solution 1964) whereas results obta ned when using homologous plasma as perfusate did not d ffer from results obtained when perfusing w th whole blood 1966

The corpuscular elements of blood seemed to be necessary for the development of the hypoxic pressor response in the present rat lung preparation. When plasma was used as perfusate from the outset and blood corpuscles lodged in the vascular bed were flushed out and discarded, no responses or only very weak responses to hypoxia were thus obtained. The perfusate, was, however not quite cell free in these experiments. White cells and blood platelets are thus readily trapped in the vascular bed of perfused lungs, and the platelets are not completely removed by flushing through the vascular bed at the onset of a perfusion (Hauge, Lunde and Waaler 1966 b)

For the development of good pressor responses to ventilation hypoxia the corpuscular elements of the blood had to be present in the perfusate for a certain period of time, and their addition to a plasma perfusate had to be carried out within the first hr of perfusion. Once the presence of blood corpuscles had resulted in the development of pressor responses to hypoxic ventilation, such responses could be demonstrated for some time also during a period of subsequent plasma perfusion.

The present studies do not tell which of the blood corpuscles are the efficient ones in causing development of the pressor response to ventilation hypoxia. Nor do we know anything about their mode of action. In further studies on the effects of the perfusate composition on this response, the presence of erythrocytes, leucocytes and thrombocytes should therefore be evaluated separately

Full equilibrium between alveolar oxygen tension and blood oxygen tension was never reached during the periods of ventilation hypoxia in the present experiments. Two factors may account for this 1) some reoxygenation can take place in the open part of the perfusion circuit and 2) some shunting of blood may occur in the lungs. Very similar curves for deoxygenation of the perfusate were obtained, however during the various tests in one and the same experiment. The changes in the hypoxic pressor responses to hypoxia within one experiment could therefore not be correlated to the level of perfusate P_{O} at precapillary or postcapillary levels.

The pulmonary arterial pressure rise on ventilation hypoxia usually started before any blood could ha e circulated from the effluent side of the preparation through the blood reservoir and into the arterial side. Likewise, the pulmonary arterial pressure started to return towards its normal level whil the influent blood was still hypoxi The hypoxic pressor response is thus apparently not due to a d rect action of low blood oxygen tension in arterial vessels.

Humoral mediators, originating from the lung tissue and/or from the corpuscular part of th blood might well be responsible for the pressor response to acut al eolar hypoxia l or further studies in this field w th selective changes in th concentration f blood corpuscles and the use of pharmacological depleters and blocking agents, isolated rat lungs might prove to be a convenient preparation.

This work has been done during tenure of Research Fellowship of the Norwegian Research Council for Science nd he Humanities Financial support from this Council, from Anders Jahres Fund for the promotion of Science and from the Norwegian Council on Cardiovascular Diseases is gratefully acknowledged

References

ALLISON, P. R., I. de B. DALY and B. A. WAALER, Bronchial circulation and pulmonary vasomotor nerve responses in isolated perfused lungs. *J. Physiol.* (Lond.) 1961. *157.* 462—474.

AVIADO, D. M., J. A. CERLETTI, J. ALANIS, P. H. BULLE and C. F. SCHMIDT. Effects of anoxia on pressure, resistance and blood (P^{32}) volume of pulmonary vessels. *Amer. J. Physiol.* 1952. *169.* 460—470.

AVIADO, D. M., J. S. LING and C. F. SCHMIDT. Effect of anoxia on pulmonary circulation: reflex pulmonary vasoconstriction. *Amer. J. Physiol.* 1957. *189.* 253—262.

DALY, I. de B. and C. HEBB, *Pulmonary and bronchial vascular systems.* Edward Arnold Ltd. London. 1966.

DALY, I. de B., D. J. RAMSAY and B. A. WAALER, Conditions governing the pulmonary vascular response to ventilation hypoxia in isolated perfused lungs of the dog. *J. Physiol.* (Lond.) 1962. *163.* 46—47P.

DALY, M. de B. and P. G. WRIGHT. The effects of anticholinesterases upon the pulmonary vascular resistance in the dog. *J. Physiol.* (Lond.) 1957. *139.* 273—293.

DUKE, H. N. Pulmonary vasomotor responses of isolated perfused cat lungs to anoxia and hypercapnia. *Quart. J. exp. Physiol.* 1951. *36.* 75—88.

DUKE, H. N. Observations on the effects of hypoxia on the pulmonary vascular bed. *J. Physiol.* (Lond.) 1957. *135.* 45—51.

DUKE, H. N. and E. M. KILLICK, Pulmonary vasomotor responses of isolated perfused cat lungs to anoxia. *J. Physiol.* (Lond.) 1952. *117.* 303—316.

EULER, U. S. v. and G. LILJESTRAND, Observations on the pulmonary arterial blood pressure in the cat. *Acta physiol. scand.* 1946. *12.* 301—320.

FISHMAN, A. P. Respiratory gases in the regulation of the pulmonary circulation. *Physiol. Rev.* 1961. *41.* 214—280.

HAUGE, A., P. K. M. LUNDE and B. A. WAALER, Vasoconstriction in isolated blood-perfused rabbit lungs and its inhibition by cresols. *Acta physiol. scand.* 1966a. *66.* 226—240.

HAUGE, A., P. K. M. LUNDE and B. A. WAALER, Some observations on trapping and release of thrombocytes in isolated perfused rabbit lungs. In *Conference on Pulmonary Circulation, Oslo 1965.* Ed. Carsten Müller. Oslo, Scandinavian University Books, Universitetsforlaget, 1966b, pp. 85—92.

IVERSEN, J. G. and J. B. STEEN, Oxygen secretion in the isolated perfused swimbladder. In *Hvalrådets Skr.* N. *48. Essays in marine physiology.* Universitetsforlaget. Oslo. 1965. 101—109.

KOVEKETT, H. and R. RÖSSLER, Versuchsanordnung zu Untersuchungen an der Bronchialmuskulatur. *Naunyn-Schmiedeberg Arch. exp. Path. Pharmak.* 1940. *195.* 71—74.

LEVY, S. and R. GATT. Isolated perfused rat lung preparation. *J. appl. Physiol.* 1966. *21.* 313—316.

LLOYD, T. C. J. Effect of alveolar hypoxia on pulmonary vascular resistance. *J. appl. Physiol.* 1964. *19.* 1086—1094.

LLOYD, T. C. J. Influence of blood pH on hypoxic pulmonary vasoconstriction. *J. appl. Physiol.* 1966. *21.* 358—364.

NISELL, O. Effects of oxygen and carbondioxide on the circulation of isolated and perfused lungs of the cat. *Acta physiol. scand.* 1948. *16.* 121—127.

NISELL, O. The action of oxygen and carbon dioxide on the bronchioles and vessels of the isolated perfused lungs. *Acta physiol. scand.* 1950. *21.* Suppl. 73.

SMITH, F. J. C. and G. A. BENNETT. The pulmonary arterial pressure in normal albino rats and the effect thereon of epinephrine. *J. exp. Med.* 1934. *59.* 173—180.

SPECTOR, W. S., *Handbook of Biological Data.* Philadelphia. Saunders. 1956. p. 279.

WAALER, B. A., A. HAUGE and P. K. M. LUNDE, Differences between the pulmonary vascular bed and vascular beds of the systemic circulation in their reaction to various stimuli. In *Conference on Pulmonary Circulation. Oslo 1965.* Ed. Carsten Müller. Oslo, Scandinavian University Books, Universitetsforlaget. 1966. 11—19.

Acta physiol. scand. 1968. 72. 45—52

From the Laboratory for the Theory of Gymnastics, University of Copenhagen, Denmark

On the Location of the Line of Gravity in Relation to L5 in Standing

By

KLAUS KLAUSEN AND BIRGER RASMUSSEN

Received 25 April 1967

Abstract

KLAUSEN, K. and B. RASMUSSEN. *On the location of the line of gravity in relation to L5 in standing.* Acta physiol. scand. 1968. *72.* 45—52.

The determination of the location of the horizontal axis in the frontal plane for movements in the sagittal plane between L4 and L5 is based on the observation that the erectores spinae muscles and the rectus abdominis muscles in the upright standing position are acting as antagonists and antigravity muscles. X-ray pictures were taken in the sagittal plane with the subjects standing a) with slight activity in the erector spinae muscles and b) with slight activity in the rectus abdominis muscles. The location of the line of gravity for the parts of the body above the level of L5 was then determined with the subjects standing in the same positions. These determinations revealed that the activity in the erector spinae muscles ceases and is replaced by activity in the rectus abdominis muscles, when the line of gravity passes a point about 3 cm dorsally to the center of the intervertebral disc between L4 and L5. Similarly the location of the line of gravity in the frontal plane was determined in relation to L5. The results are discussed in view of previous findings.

The activity of the trunk muscles in a normal symmetrical standing position has previously been investigated by several authors, *e.g.* Floyd and Silver 1955, Joseph and McColl 1961, Portnoy and Morin 1956, Asmussen 1960, Klausen 1965. On the whole these investigations have revealed that the upright position of the spine is maintained by activity in one set of muscles only, either in the erector spinae muscles (in most cases) or in the abdominal wall muscles (mainly rectus abdominis). It is, therefore, believed that in the upright position of the spine an equilibrium exists between the force of gravity on one side, and the muscular activity on the other side of the horizontal axes in the frontal plane for movements in the sagittal plane between the vertebrae.

One critical point in this concept is that it is difficult to establish the exact location of these axes between the vertebrae. Dittmar (1930) found that the axes in the lumbar part of the spine always were located dorsally to the center of the discus. Wiles (1935) found that the axes were located even more dorsally just ventral to the articular processes. In other investigations the axes have been assumed to be located at the

center of the discs on the ground that the discs (and the vertebrae) constitute the weight bearing elements of the spine.

The aim of the present experiments is to determine the location of the frontal axis of movement between L4 and L5. The determination is based on the assumption that the erector spinae and rectus abdominis muscles are acting as antagonists and antigravity muscles. According to this the location of the axis should be identical with the location of the line of gravity for the parts of the body above L5 just at the moment when the activity in the erector spinae muscles ceases and is replaced by activity in the rectus abdominis muscles during a slight leaning backwards form normal standing position.

Methods

15 young healthy subjects were used for the experiments. They were barefooted and wore bathing suit during the investigation. Each experiment was carried out in two stages.

A. Three X-ray pictures of the spine were taken with the subject in the standing position. The activity of the trunk muscles was registered, simultaneously with the taking of the X-ray pictures, by means of surface electrodes. The electrodes consisted of two small silver plates of the same type as described by Klausen (1965) except that they had been modified in such a way that they could be pasted [illegible] waterproof to the skin over the muscles. The action potentials were amplified and recorded with a DISA-electromyograph, and the EMG were "integrated" to give the average of the numerical value of the voltage by means of a *DISA MEAN VOLTAGE UNIT type 13Ba03*.

The following trunk muscles were investigated: 1) The erectores spinae at the level of L4 and L5 as close to the medial plane as possible. 2) The rectus abdominis just above the umbilicus, and 3) The obliquus abdominis externus about 2—4 cm above the highest point of crista iliaca. All electrodes were placed as nearly as possible parallel with the muscle fibres.

The first X-ray picture of the spine was taken in the frontal plane with the subject standing in his habitual, symmetrical position. The distance between the heels was adjusted to 3—10 cm and the angle between the feet to 25—40°. The subject was asked to look straight ahead, and the hands were hanging at the sides (this position is called pos. 1). The activity of the left and right erector spinae muscles was recorded simultaneously with the taking of the X-ray picture.

The second picture of the spine was taken in the sagittal plane with the subject standing in the same position as described above except for the hands, which were crossed in front of the abdomen so that the lumbar part of the spine could be exposed to the X-rays (pos. 2). The activity of the left erectores spinae and rectus abdominis muscles was recorded simultaneously.

All subjects showed slight to moderate activity only in the erector spinae muscles in pos. 2. After the second X-ray picture was taken the subject was asked to lean slightly backwards, and try to assume a position where the activity in the erectores spinae muscles has just ceased and is replaced by a slight activity in the rectus abdominis muscles. The subject was able to control this position by following the action potentials from the muscles on the monitor [illegible] of the DISA-electromyograph. The third picture of the spine was taken when the subject had learned to assume the right position (pos. 3), usually after only a few trials.

In each determination in pos. 2 with main activity in the erector spinae muscles, a small activity (2—5 μV) could always be registered in the rectus abdominis muscles, and in pos. 3 with main activity in the rectus abdominis muscles a small activity could be measured in the erector spinae muscles. This activity [illegible] could be shown to be due to the noise level of the DISA amplifiers. Hence the [illegible] amplitudes (2—5 μV) [illegible] no indication of muscular activity.

B. After the X-ray pictures were taken the location of the line of gravity for the part of the body above L5 was determined, using a modification of the method, described by Klausen 19[illegible].

The experimental set up is shown in Fig. 1, which shows a longitudinal section of the platforms and a subject standing on the upper platform. An angle-iron forms an axis on one end of the platform (ax. and [illegible]) and the weight bearing point of the platforms rests on strain gauge dynamometer (st. and [illegible]).

The weight of the subject (G) is determined by the sum of the pressures exerted on the dynamometers (P + P[illegible]) and the position of the line of gravity in relation to [illegible] is calculated from the equation $G \times a = P \times b$, where b is the distance from [illegible] to the weight bearing point on st. and a is the distance from [illegible] to the line of gravity. The weight determination by means of the platforms is highly accurate although the accuracy is dependent on the size of the load. Thus 62

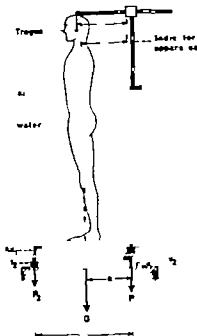


Fig 1 Schematic drawing of subject standing on the platforms, used for the determination of the weight and of the location of the line of gravity for the parts of he body bov the level of L5 in relation to tragus. See text.

calibrations with load of 10 kg revealed standard deviation of bout 3 per cent while standard deviation of only 0.3 per cent was found when he platforms were calibra ed with load of 35 kg. This difference in accuracy is probably due to the fact that the platforms do not const ut complete rigid system

As can be seen from Fig 1 the subjects stood on the platforms in w er to bout the level of the discus between L4 and L5 The pressure exerted on the platforms is, therefore only due to the parts of the body abov the water (Åkerblom 1948) provided that th specific gravity of the lower extremities and water is the same (see Discussion) The location of the line of gra ity for the parts of the body bov the w ter in relation t tragus — -a, where is the horizontal distance from tragus to ax which is measured by means of an indicator pparatus (see Fig 1 The location of he gra ity line in the frontal plane was similarly determined in relation to processus spinosus of the 7 ervical ertebra.

Three sets of gra ity line determinations were performed during which the subject ttempted assume the same position as he did when the X-ray pictures were taken (pos. 1—pos. 3) The 3 positions were reproduced by means of the action potentials from the muscles seen on the monitor cathode ray tubes. 2—7 determinations of the location of the line of gravity were mad each of the 3 positions.

The gravity line determinations were transferred t the corresponding X-ray pictures on which tragus (and proc. spin. on 7 ertebra) had been marked by means of small circula piece of lead. The location of the line of gra ity in the sagittal plane was determined in relation to he center of he discus between L4 and L5 (Fig 2 and Fig. 3) and the location of the line of gravity in he frontal plane as determined in relation to the center of the discus between L5 and S 1 Fig 4

Results

Characteristics of th subjects are given in Table I The height and total body weight are measured with traditional measuring apparatus. The weight of the head, arms nd trunk above th level of L5 presented for each subject, represent the average of t least 10 determinations on the platform. The standard deviation of the average



Fig 2 Fig. 3 Fig 4

Fig 2. Sagittal X-ray picture of subject during normal standing (pos. 2) Not the location of the line of gra ity in relation t L4 nd L5

Fig. 3 Sagittal X-ray picture of subject leaning slightly backwards (pos. 3) Not the location of the line of gra y in relation to L4 and L5

Fig 4 Frontal X-ray picture of subject during normal standing (pos. 1) Note the loca ion of the ine of gra ity relation to L5.

for each subject is about 1 . This is somewhat more than found when the platforms are cal brated with 35 kg (SD = 0.3 per cent) and is probably due to the small postural oscillations back and forth of the subjects when standing on the platform.

Fig 5 gives a graphic presentation of all the determinations in the sagittal plane The position of the lin of gra ity in relation to the center of the discus between L4 and L5 is gi en on the abscissa, and the corresponding mean activity of the trunk muscl s is shown on the ordinate

The determinations made with the subjects standi g in pos. 2 (normal standing) can be recognized by main acti ity in the erector spinae muscles (black dots) and as can be seen the line of gravity is located dorsally to the center of the discus in 26 of 29 determinations in this position. Pos. 3 (leaning backwards) is characterized by main activity in the rectus abdominis muscles (circles) and in Fig 5 it s seen that this first occurs when the line of gravity is located 3—4 cm d to the center of the discus between L4 and L5

TABLE I Characteristics of subjects

Subjects	Height cm	Age years	Total body weight kg	Weight of the body above L5 kg	% of total
Females					
ES	153.0	24	54.5	26.3	48.3
BT	169.0	23	67.8	32.0	48.6
VS	165.4	25	52.0	23.9	45.9
LH	163.5	24	55.3	25.1	45.5
GL	161.0	22	62.0	28.8	46.4
TB	170.5	21	65.0	24.7	38.0
Mean	163.7	23.2	59.4	26.8	45.5
Males					
JB	182.0	22	63.9	32.6	48.8
JBH	178.1	21	64.2	35.2	54.8
VKJ	172.1	27	74.0	38.9	52.5
EJ	171.1	23	78.5	39.0	49.9
NGK	175.4	21	67.9	33.3	49.0
JM	174.5	20	70.5	34.7	49.2
KK	184.0	32	73.0	37.5	51.3
BR	168.0	30	72.0	32.7	45.4
RM	182.0	21	71.0	36.7	51.6
Mean	176.4	24.1	70.8	35.6	50.3

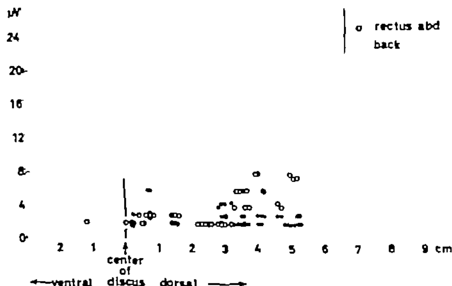


Fig. 5. Graph showing the values for mean electrical activity (ordinate) in the erector spinae muscles (●) and in the rectus abdominis muscles ( ) plotted against the distance of the line of gravity in the sagittal plane from the center of the discus between L4 and L5 (abscissa)

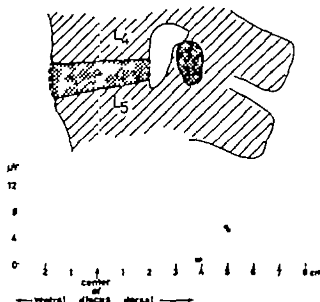


Fig 6. Selected data from Fig 3, in which the activity in the erector spinae muscles and the rectus abdominis muscles was the same during the X-ray photographing and during the determination of the line of gravity. Same symbols as in Fig 5. See text.

A comparison of the activity of the trunk muscles found in each subject during the gravity line determinations with the muscle activity registered simultaneously with the X ray photographing revealed that the subjects not always were able to reproduce the same positions in the two stages of the experiments. However it was possible to select at least one determination on each subject in which the activity during the determination of the gravity line in pos. 2 and pos. 3 was identical (± 1 μV) with the activity found during the X ray photographing. These selected determinations are shown in Fig 6. In the figure the activity of the muscles is corrected for the noice level mentioned before. The average size and position of L4 and L5 is schematically shown in the figure. The activity in the erector spinae muscles ceases and is taken over by the rectus abdominis muscles, when the line of gravity passes a point about 3 cm dorsal to the center of the discus between L4 and L5, and as can be seen on fig 6 this point corresponds to the location of the joints between the interarticular processes.

In normal standing (pos. 2) the line of gravity is on an average located 1.26 cm dorsal to the center of the discus between L4 and L5. This is in line with previous experiments (Klausen 1965).

The location of the line of gravity in the frontal plane was determined in 12 of the subjects and at least 3 determinations were made on each subject. No correlation was seen between the activity in left and right trunk muscles and the location of the line of gravity in relation to L5. The average of all determinations on all the subjects gave a location of the line of gravity in relation to the medial plane through L5 of 0.65 cm to the right (SEM = ± 0.15 cm).

Discussion

In the determination of the weight of the parts of the body above the level of L5 it has been assumed that the density of the parts of the body under water is identical with the density of water this will introduce an error in the determinations, since the density of water in the present experiments was found to be close to 1 00 while according to the literature an average density for both sexes of the lower parts of the body has been estimated to about 1 05 For females it may be as low as 1 04 and for males as high as 1 06 (Rathburn and Pace 1945 Brozek *et al.* 1963 Brozek 1965) Thus the weight of the body above L5 presented for the two sexes in Table I on the average are overestimated with about 1.5 kg ± 1 per cent. In the standing position, however the subjects stood with the hands under water A few weight determinations on two of the subjects with a) hands under water and b) hands over water revealed *that the weight of the hands is about 1.0—1.5 kg which is in line with* anthropometric data available in the literature (Carlsöö 1966) The underestimation of the weight of head, arms and trunk above the level of L5 due to the weight of the hands thus compensates for the above described overestimation.

The weight determinations for head arms and trunk are of interest, since very few data are available in the literature. In publications dealing with calculations of the external forces acting on the intervertebral discs (*e g* Nachemson 1965) the weight of the body above the level of L5 has been estimated from Ruff's data (1950) Ruff identified this weight with the pressure necessary to compress the spine in a horizontal position to the same length as in a free standing position. He found that it amounted to 60 per cent of the total weight of the subjects (males) This is 10—15 per cent more than in the present determinations, and the explanation is probably that he did not take into account the compressive effect of the postural muscles and ligaments on the spine.

The above mentioned two main errors in the weight determination will have an influence on the determination of the line of gravity in relation to the intervertebral discs between L4 and L5 A correction for the specific gravity of the parts of the body under water will on *an average move the line of gravity 2—3 mm forwards, but this* is completely compensated by a correction for the weight of the hands, which, provided the torque of the trunk muscles is the same during the X-ray photographing and the gravity line determination, will move the line of gravity on an average 3—4 mm backwards. Consequently no corrections have been made in Fig 5 and *Fig. 6*

Another source of error and probably the most important one is that the experiments were carried out in two stages. Thus it is possible that the standing position of a subject during the X-ray photographing is different from the position during the gravity line determinations. This will certainly be true for some of the determinations presented in Fig 5, since the EMG s from the muscles were not always the same at the two occasions. In Fig 6 however the electrical activity in the postural muscles at the two stages of the experiments is quantitatively identical This should give a good justification for assuming that in these selected determinations the

orientation of the spine has been the same in the two stages of the experiments. It seems, therefore, verified that the shift in activity from the erector spinae muscles to the rectus abdominis muscles takes place when the line of gravity passes a point about 3 cm dorsally to the center of the discus between L4 and L5, *i.e.* when the line of gravity passes the joints between the articular processes.

The interpretation of this finding is rather difficult. If we suppose that the conclusion from previous experiments (mentioned in the introduction) is true, namely that the trunk muscles are acting as antigravity muscles, then the conclusion of the present experiments will be that the horizontal axis in the frontal plane for small movements in the sagittal plane must be located in plane with the interarticular joints. This is in line with Wiles' results from 1935 but does not agree with Dittmar's experiments from 1930. As pointed out by Klausen (1965) a location of the axis through the interarticular joints will cause a minimum of stress on these joints during flexion or extension of the spine in the sagittal plane. The difficulty, however, is to explain how the interaction between the force of gravity and the muscle activity in normal standing position can take place around the rather weak interarticular joints, although most of the external forces acting on the spine has to be transferred to the weight bearing parts of the system, *i.e.* the corpora and the intervertebral discs. A satisfactory explanation of this problem can not be given at the moment, but will have to await further investigations.

The authors want to express their gratitude to the Testing and Observation Institute of The Danish National Association for Infantile Paralysis, where most of this investigation was performed.

References

ÅKERBLOM, B. *Standing and sitting posture.* Stockholm, 1948. 187

ASMUSSEN, E., The weight-carrying function of the human spine. *Acta orthop. scand.* 1960. 276—290.

BROZEK, J., F. GRANDE, J. T. ANDERSON and A. KEYS, Densitometric analysis of body composition. Revision of some quantitative assumptions. *Ann. N. Y. Acad. Sci.* 1963. *110.* 113—140.

BROZEK, J. *Human body composition.* Pergamon Press Ltd. Oxford 1965.

CARLSÖÖ, S., *Antropometri.* Handbook i ergonomi, 1966. p. 214—241 Stockholm.

DITTMAR, O. Die sagittal- und lateralflexorische Bewegung der menschlichen Lendenwirbelsäule im Röntgenbild (Zur Mechanologie der Wirbelsäule. 1. Mitteilung). *Z. Anat. Entwickl. Gesch.* 1930. *92.* 644—667.

FLOYD, W. F. and P. H. S. SILVER. The function of the erectores spinae muscles in certain movements and postures in man. *J. Physiol.* (Lond.) 1955. *129.* 184—203.

JOSEPH, J. and MCCOLL. Electromyography of muscles of posture: Posterior vertebral muscles in males. *J. Physiol.* (Lond.) 1961. *157.* 33—37.

KLAUSEN, K., The form and function of the loaded human spine. *Acta physiol. scand.* 1965. 65. 176—190.

NACHEMSON, A., The effect of forward leaning on lumbar intradiscal pressure. *Acta orthop. scand.* 1965. 35. 314—328.

PORTNOY, H. and F. MORIN. Electromyographic study of postural muscles in various positions and movements. *Amer. J. Physiol.* 1956. *186.* 122—126.

RATHBUN, E. N. and N. PACE, Studies on body composition. I. The determination of total body fat by means of the body specific gravity. *J. biol. Chem.* 1945. *158.* 667—676.

RUFF, S., Brief acceleration: Less than one second. I. *German Aviation Medicine. World War II.* 1950. I. 584—597.

WILES, P. Movements of the lumbar vertebrae during flexion and extension. *Proc. roy. Soc. Med.* 1935. 28. 647—651.

Acta physiol. scand. 1968. 72. 53—61

From the Department of Pharmacology University of Göteborg, Sweden

A Comparison of the Subcellular Sites of Action of Reserpine and Benzquinamide

By

Per Lundborg and Robert E. Stitzel

Received 28 April 1967

Abstract

P Lundborg and Stitzel, R. E. *A comparison of the subcellular sites of action of reserpine and benzquinamide* Acta physiol. scand. 1968. 72 53—61

Both reserpine and benzquinamide were capable of impairing the in vitro uptake of C¹ adrenaline by bovine and rabbit adreno-medullary granules, however they differed markedly in their relative potency The in vivo studies using mice and the in vivo — in vitro studies employing rabbits indicated that the time course of granular blockade after reserpine and benzquinamide injections also differed. Benzquinamide was shorter-acting and less potent than was reserpine Subcellular distribution studies indicated that while both drugs could impair the in vivo uptake of ^{3}H-α-methyl noradrenaline, only reserpine administration resulted in both an increase in the amount of ^{3}H-α-methyl noradrenaline found in the supernatant fraction and an increase in the amount of ^{3}H-α-methyl normetanephrine recovered from the whole heart It is concluded that while reserpine and benzquinamide have many qualitatively similar effects on amine uptake and depletion, differences in their actions on the subcellular level can be uncovered.

Benzquinamide is a benzoquinolizine derivative which appears to have several properties in common with reserpine These include the ability to disrupt conditioned avoidance responses (Weissman and Finger 1962 Andén and Hanson 1966) and to decrease central and peripheral catecholamine stores (Pletscher Brossi and Gey 1962, Weissman and Finger 1962 Andén and Hanson 1966) However it has been shown (Carlsson and Lindqvist 1966) that pretreatment of mice with benzquinamide enhances rather than protects animals against the depleting action of reserpine On this basis it was suggested that the site of amine depletion produced by benzquinamide was different from that of reserpine It was of interest, therefore, to examine the effects of these two agents in greater detail. The present studies were performed to compare the effects of benzquinamide and reserpine on the uptake of amines both in vivo and in vitro

Methods

The experiments were performed using white female mice weighing about 20 g and on rabbits of both sexes, weighing about 2.5 kg. Benzquinamid (100 mg/kg) or reserpine (5 mg/kg) were injected i.v. into rabbits, while mice received 50 or 100 mg/kg of benzquinamide and 10 mg/kg of reserpine. The animals were killed at various intervals after the injection. The experiments using mice were performed at a temperature of 29° C and those on rabbits at room temperature (23—24°). The catecholamine content of rabbit adrenal extract was determined by the method of Bertler, Carlsson and Rosengren (1958).

In vitro granule experiments. Cow or rabbit adrenal medullae were homogenized with a loose-fitting plastic pestle in 0.3 M sucrose. Unbroken cells and nuclei were removed by centrifugation at 800 × g for 5 min. The supernatant thus obtained was decanted and centrifuged at 26 000 × g for 20 min. The granules were then suspended in 0.3 M sucrose. Amine granules from bovine adrenal medulla were prepared and incubated essentially as described by Hillarp (1958) and Carlsson, Hillarp and Waldeck (1963). Rabbit adrenal medullary granules were prepared and incubated as described by Carlsson, Hillarp and Waldeck (1963) and Lundborg (1963). Incubations were performed without shaking at 0° and 31° for 30 min.

In vivo experiments. Mice divided into groups of 6 were given either reserpine (10 mg/kg) or benzquinamide (50 or 100 mg/kg) at varying time intervals before the i.v. injection of ³H-α-methyl noradrenaline (100 μg/kg). All animals were killed 15 min after the injection of the labelled amine. Hearts were removed and homogenization performed in an ice bath using a plastic pestle. A coarse fraction was obtained by centrifugation of the homogenate in the cold at 2000 × g for 10 min. The supernatant obtained was then centrifuged at 100 000 × g for 60 min in a Spinco Model L Ultracentrifuge providing two more fractions, particulate (sediment) and high speed supernatant. Details of the subcellular fractionation and amine isolation procedures have been described previously (Stitzel and Lundborg 1967, Carlsson and Waldeck 1963).

Substances used. ³H-α-methyl noradrenaline was prepared by the research laboratory of Hässle Ltd in cooperation with this department (Hallhagen and Waldeck, to be published). C¹⁴-adrenaline was obtained from New England Nuclear Corp. Reserpine and benzquinamide were generously supplied by Swedish Ciba Ltd and Swedish Pfizer Ltd.

Results

Influence of reserpine and benzquinamide on the in vitro uptake of C¹⁴-adrenaline by bovine adrenal granules. Reserpine, even in relatively low concentrations (1 × 10⁻⁷M), markedly impaired the accumulation of C¹⁴ adrenaline by bovine medullary granules (Table I). On the other hand, incubation of bovine granules in the presence of benzquinamide resulted in an appreciable impairment of uptake only when a concentration of at least 1 × 10 M was used (Table I). Both compounds impaired uptake mechanisms in isolated granules, but they differed markedly in their relative potency.

TABLE I. Influence of benzquinamide and reserpine on the uptake of ¹⁴C-adrenaline by bovine adrenal granules

Compound	Conc. of compound (M)	Per cent Inhibition	Number of experiments
Benzquinamide	5 10	14.9 ± 0.94	3
Benzquinamid	1 10	57.1 ± 4.62	5
Benzquinamide	5 10	78.7 ± 4.98	5
Reserpine	1 10	36.9 ± 3.96	4
Reserpine	1 10	60.8 ± 4.73	4
Reserpine	1 10	93.8 ± 2.81	4

The final concentration of adrenaline (labelled plus unlabelled) was 3 × 10 M.

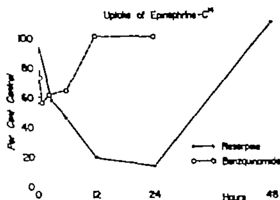


Fig. 1. *In vitro* uptake of C -adrenaline by adrenal medullary granules isolated at various intervals following the injection of either reserpine (5 mg/kg) or benzquinamide (100 mg kg) to rabbits.

Effects of reserpine and benzquinamide pretreatment on catecholamine uptake in granules isolated from rabbit adrenal medulla. Rabbits were given either reserpine or benzquinamide and at different intervals after drug administration (1/2—48 hrs) the animals were killed by an i.v. injection of air. The adrenal glands were removed and granules were prepared. After incubation aliquots were taken both for liquid scintillation counting and for measurement of endogenous catecholamine content. In each experiment one reserpine or benzquinamide treated and one control animal were used. Fig. 1 shows the amount of C[1] -adrenaline which was incorporated into the granules obtained from these animals. The values are expressed as uptake in percent of the control value.

Reserpine caused a pronounced blockade of the uptake of adrenaline by the amine-containing granules. This effect was evident for up to 24 hrs after drug injection uptake then returned to almost normal values by 48 hrs (Fig. 1). Rabbits who had been pretreated with 100 mg/kg of benzquinamide also showed an impairment of C[1] -adrenaline uptake, but this effect had disappeared about 12 hrs after drug administration (Fig. 1). The maximum impairment of amine uptake produced by benzquinamide was approximately 45 per cent while that produced by reserpine was about 85 per cent. It is apparent that both drugs, when given *in vivo* impaired catecholamine uptake mechanisms present in rabbit adrenal granules, but the time course of their actions was quite different (Fig. 1)

Effect of erpine and benzquinamid pret atment on amin levels abbit adrenal gland. Pretreatment with either benzquinamide or reserpine resulted in a loss of catecholamines from the rabbit adrenal gland (Fig. 2). Amine loss following benzquinamide administration was more transient than that which occurred after reserpine. The maximal amine deficit (70 per cent) which resulted from benzquinamide pretreatment was reduced to about 20 per cent by 4 hrs following its injection, while reserpine treatment produced a more marked (100 per cent) and

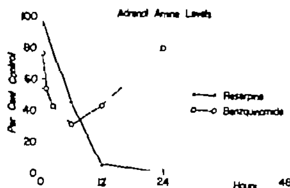


Fig. 2. Effect of reserpine (5 mg/kg) and benzquinamide (100 mg/kg) on rabbit adrenal medullary catecholamine levels.

more prolonged amine loss (Fig. 2). A comparison of Fig. 1 and 2 shows that after 12 hrs (benzquinamide) or 48 hrs (reserpine) the uptake of C^{14} adrenaline was restored to approximately normal values while the endogenous levels of catecholamines in the adrenal medulla were still very low.

Effect of reserpine and benzquinamide on the uptake of H^3-α methyl noradrenaline (^{3}H-α MeNA) by subcellular fractions of the mouse heart. Mice given reserpine showed a greatly diminished uptake of ^{3}H-α MeNA in the particulate fraction isolated from heart muscle (Table II). This was accompanied by an appreciable accumulation of tritiated amine in the supernatant fraction. The latter accumulation was particularly marked at the early intervals. Benzquinamide at a dose of 100 mg/kg also impaired the uptake of ^{3}H-α-MeNA into the particulate fraction, but

TABLE II. Uptake of ^{3}H-α-methyl noradrenaline (^{3}H-α-MeNA) in subcellular fractions of the mouse heart after treatment with reserpine

Treatment	Time after drug administration	Number of experiments	^{3}H-α-MeNA ng/g	
			Particulate	Supernatant
Control	—	6	29.47 ± 1.76	39.66 ± 1.08
Reserpine	1	4	8.13 ± 0.80	55.69 ± 2.69
(10 mg/kg)	1	4	8.18 ± 0.87	64.66 ± 9.31
	2	4	8.69 ± 0.87	70.35 ± 4.47
	4	4	7.67 ± 0.55	89.88 ± 5.05
	12	2	8.32 ± 0.53	70.56 ± 2.20
	24	4	12.87 ± 0.80	[illegible]
	48	4	18.92 ± 0.58	51.13 ± 2.31
	96	4	27.43 ± 2.03	49.07 ± 2.69

Control animals received ^{3}H-α-MeNA alone. All animals were sacrificed 15 min after receiving the labelled amine. The values given for ^{3}H-α-MeNA content are means ± S.E.M. Each experiment was performed on 6 pooled hearts.

TABLE III Uptake of ^{3}H-α-methyl noradrenaline (^{3}H-α-MeNA) in subcellular fractions of the mouse heart after treatment with benzquinamide

Treatment	Time after drug administration (hrs)	Number of experiments	^{3}H-α-MeNA-ng/g Particulate	^{3}H-α-MeNA-ng/g Supernatant
Control	—	7	29.24 ± 1.69	36.73 ± 0.54
Benzquinamide	1/2	4	11.88 ± 1.4	31.91 ± 2.37
(50 mg/kg)	1	4	25.68 ± 2.07	32.55 ± 2.89
	2	4	26.44 ± 1.77	33.73 ± 3.17
	4	4	26.46 ± 1.53	33.69 ± 1.68
Benzquinamide	1/2	4	19.57 ± 0.73	32.25 ± 0.77
(100 mg/kg)	1	4	21.84 ± 2.93	30.88 ± 1.6
	2	4	27.54 ± 0.98	32.13 ± 0.67
	4	4	27.18 ± 0.65	32.70 ± .53

Control animals received ^{3}H-α-MeNA alone. All animals were sacrificed 15 min after receiving the labelled amine. The values given for ^{3}H-α-MeNA content are means ± S.E.M. Each experiment was performed on 6 pooled hearts.

this blockade was not as marked nor was it accompanied by a concomitant increase in ^{3}H-α-MeNA levels in the supernatant fraction (Table III). Reserpine-induced granule blockade lasted between 48 and 96 hrs while that produced by benzquinamide had essentially disappeared after ? hrs.

Distribution of ^{3}H-α-methyl normetanephrine (^{3}H-α-MeNM) formed from exogenously administered ^{3}H-α-MeNA after pretreatment with reserpine or benzquinamide. After reserpine but not after benzquinamide there was a marked increase in the appearance of the O-methylated metabolite of ^{3}H-α-MeNA,

TABLE IV Formation of ^{3}H-α-methyl normetanephrine (^{3}H-α-MeNM) from exogenously administered ^{3}H-α-methyl noradrenaline following pretreatment with benzquinamide or reserpine

Treatment	Time after drug administration (hrs)	Number of experiments	^{3}H-α-MeNM-ng/g
Control	—	8	20.50 ± 0.85
Benzquinamide (50 mg/kg)	1/2	3	19.35 ± 1.33
	1	4	21.98 ± 1.4
	2	4	23.5 ± 2.1
Reserpine (10 mg/kg)	1/2	4	38.97 ± 2.51
	1	4	37.88 ± 2.44
	2	4	33.17 ± 1.48

Both control and drug pretreated animals were sacrificed 15 min after receiving an injection of ^{3}H-α-methyl noradrenaline. The values given for ^{3}H-α-MeNM content are means ± S.E.M. Each experiment was performed on 6 pooled hearts.

TABLE V Effect of benzquinamide and reserpine alone and in combination on the uptake of ³H-α-methyl noradrenalin (³H-α-MeNA)

Drug	Number of experiments.	³H-α-MeNA/ng/g	
		Particulat	Supernatant
None	11	28.99 ± 1.20	38.69 ± 0.70
Benzquinamide (100 mg/kg)	4	19.57 ± 0.75	32.25 ± 0.77
Reserpine (10 mg/kg)	4	7.67 ± 0.35	89.88 ± 5.05
Benzquinamid + Reserpine	4	7.14 ± 0.38	60.06 ± 2.25

The values given for ³H-α-MeNA content are means ± S.E.M. Each experiment was performed on 6 pooled mouse hearts. Benzquinamid was given 1/2 hr and reserpine 4 hr before the i.v. injection of ³H-α-MeNA. All animals were sacrificed 15 min after administration of the labelled amine.

³H-α-MeNM (Table IV) Pretreatment with reserpine resulted in approximately a doubling while benzquinamide administration caused only a slight increase in the amounts of ³H-α-MeNM that could be recovered from heart tissue following an intravenous injection of ³H-α MeNA.

Effects of benzquinamide and reserpine alone and in combination on the uptake of ³H-α MeNa by subcellular fractions of the mouse heart Four hrs after its administration reserpine markedly impaired the uptake of ³H-α MeNA by the particulate fraction and caused a large increase in the amount of labelled amine found in the supernatant fraction (Table V) Benzquinamide (100 mg/kg) treatment resulted in a much smaller granular blockade and caused only a reduction in the amount of ³H-α MeNA found in the supernatant fraction (Table V) A combination of the two agents did not enhance reserpine's granular blockade but did decrease the elevated amount of tritiated amine normally found in the supernatant fraction after reserpine administration (Table V)

Discussion

Benzquinamide administration results in changes in behavioural patterns which similar to those seen after a single injection of reserpine These include a moderate to heavy sedation and a disruption of certain conditioned avoidance responses (Weissman and Finger 196 Andén and Hanson 1966) Seiden and Carlsson (1964) and Andén and Hanson (1966) have suggested that reserpine and benzquinamide produce their disruption of conditioned avoidance responses through similar mechanisms, namely a depletion of catecholamines.

It has been shown that reserpine has the ability to inhibit an ATP Mg dependent mechanism present both in the adrenal medulla (Kirshner 1962 Carlsson, Hillarp

and Waldeck 1963) and in adrenergic nerves (Lindmar and Muscholl 1964 Euler and Lishajko 1965 Stitzel and Lundborg 1967) and apparently it is this mechanism which is responsible for the uptake and retention of monoamines in sympathetically innervated tissue It was of interest, therefore, to see if reserpine and benzquinamide, compounds which had parallel effects on certain behavioral responses, produced their amine-depleting effects through similar mechanisms.

Benzquinamide when given *in vivo* to rabbits can impair uptake mechanisms present in subsequently isolated adrenal granules for a period of about 6 hrs, while reserpine-induced blockade is evident for up to 24 hrs. Furthermore, both drugs are capable of lowering the catecholamine content of the rabbit adrenal gland but the time course of depletion and repletion for each drug are different. Adrenal amine content has returned to about 85 per cent of normal 24 hrs after benzquinamide administration, while amine levels in reserpine-treated animals are only 15 per cent of control values at this time.

From these results it is apparent that benzquinamide and reserpine effect qualitatively similar changes in amine uptake and storage mechanisms, but the effects produced by benzquinamide are less pronounced and are of a shorter duration. In these respects benzquinamide appears to resemble tetrabenazine, a compound which is closely related chemically (Quinn, Shore and Brodie 1959) The site of action of benzquinamide probably differs from that of tetrabenazine, however since Carlsson and Lindqvist (1966) have shown that the latter but not the former compound, can protect rabbits and mice against the actions of reserpine on certain behavioural parameters and on brain 5-HT levels. This conclusion is further supported by the observation that after pretreatment with the monoamine oxidase inhibitor nialamide, tetrabenazine causes excitation (Pletscher Brossi and Grey 1962) while benzquinamide causes sedation (Weissman and Finger 1962)

The time course of action of benzquinamide is probably better correlated to the tenure of the drug in the body than is that seen after reserpine. It is interesting to note that in our studies the inhibition of uptake produced by benzquinamide is maximal at 1 hr and then declines. This may be correlated with the findings of Weissman and Finger (1962) which showed that the concentration of benzquinamide in body tissues falls to almost unmeasurable levels 1 hr after its injection. The relatively rapid disappearance of inhibition observed after benzquinamide treatment probably indicates the ready reversibility of its action. The rather prolonged impairment of amine uptake and lowering of amine content seen after reserpine is in agreement with studies indicating that reserpine acts irreversibly by damaging an amine-binding mechanism in adrenergic granules (Hess, Shore and Brodie 1956) Repletion after reserpine administration probably does not occur until new granules are formed (Dahlström and Häggendal 1967)

The *in vitro* data obtained from studies using bovine adrenal medullary granules indicates that both benzquinamide and reserpine are effective inhibitors of catecholamine uptake although they differ by almost 1000-fold in their potency The ability of benzquinamide to inhibit amine uptake *in vitro* argues against the possibility h-

its *in vivo* effects are due to the action of a metabolic product (Weissman and Finger 1962). However the possibility cannot be excluded that the de-acetylated product is also an inhibitor of amine uptake and therefore may partly contribute to an *in vivo* effect.

Benzquinamide like reserpine is an effective agent in impairing the uptake of ^{3}H-α MeNA *in vivo*. Both reserpine and benzquinamide caused a diminished retention of ^{3}H-α MeNA in the particulate fraction of the mouse heart, although reserpine was more potent in this respect. Reserpine produced a long-lasting (96 hrs) impairment of amine uptake into adrenergic granules, while the effect seen after benzquinamide administration was essentially gone after 2 hrs. These results are in agreement with our *in vivo-in vitro* studies employing rabbits and with previous findings which also demonstrated the relatively short duration of action of benzquinamide (Carlsson and Lundqvist 1966, Andén and Hanson 1966).

After reserpine administration there was a pronounced increase in the concentration of ^{3}H-α MeNA recovered from the supernatant fraction while benzquinamide produced a slight decrease only. It is possible that although both drugs can impair uptake mechanism in the particulate fraction, their actions on the cytoplasmic fraction differ. Benzquinamide may enhance the loss of ^{3}H-α-MeNA from this fraction. This is supported by the finding that a combination of reserpine and benzquinamide causes no further impairment of amine uptake in the particulate fraction but does reduce the elevated ^{3}H-α-MeNA levels found in the supernatant fraction after reserpine alone.

Pretreatment of the animals with reserpine caused a pronounced increase in the amount of ^{3}H-α MeNMN recovered from the tissues. This increase was probably due to the decreased capacity of the adrenergic nerves to bind ^{3}H-α-MeNA, thus exposing it to enzymatic destruction by catechol-O-methyl transferase. Benzquinamide pretreatment however caused only a minimal elevation of ^{3}H-α-MeNMN levels. This was, in all probability correlated with the smaller impairment of amine storage capacity produced by benzquinamide.

Häggendal and Roos (1967) studying endogenous normetanephrine levels have reported only a transient decrease following benzquinamide administration. A simultaneous decrease in NA and dopamine levels accompanied by an increase in the levels of the acid metabolites, dihydroxyphenylacetic acid and homovanillic acid, have been demonstrated following both reserpine (Andén, Roos and Werdinius 1964) and benzquinamide (Häggendal and Roos 1967). The latter observations support the assumption that both drugs release endogenous amines and probably do not impair catecholamine synthesis.

The results of the present study indicate that benzquinamide, at least in higher doses, produces qualitative changes in catecholamine uptake and storage which are similar to those seen after reserpine. However benzquinamide has a much shorter and less pronounced action and its effects appear to be readily reversible. Furthermore the subcellular distribution studies indicate that the actions of the two drugs are not identical in all respects.

The research reported in this manuscript has been supported by the Swedish State Medical Research Council (B 67 14X 155-03 A) and the Faculty of Medicine, University of Göteborg, Sweden. RES would like to thank West Virginia University for the leave of absence which allowed him to carry out this work, and the SSMRC for their financial support. The highly competent technical assistance of Miss Lena Ramstedt is gratefully acknowledged.

References

Andén, N. E. and L. Hanson, Effect of benzquinamide on conditioned avoidance response and brain monoamine levels in the cat. *Psychopharmacologia* (Berl.) 1966 *10* 110—115.

Andén, N. E., B. E. Roos and B. Werdinius, On the occurrence of homovanillic acid in brain and cerebrospinal fluid and its determination by a fluorometric method. *Life Sci.* 1964 *3* 149—158.

Bertler, A., A. Carlsson and E. Rosengren, A method for the fluorimetric determination of adrenaline and noradrenaline in tissues. *Acta physiol. scand.* 1958 *44* 273—292.

Carlsson, A., N.-A. Hillarp and B. Waldeck, Analysis of the Mg++ ATP dependent storage mechanism in the amine granules of the adrenal medulla. *Acta physiol. scand.* 1963 *59* Suppl. 215 1—38.

Carlsson, A. and M. Lindqvist, The interference of tetrabenazine, benzquinamide and prenylamine with the action of reserpine. *Acta pharmacol.* (Kbh.) 1966 *24* 112—120.

Carlsson, A. and B. Waldeck, On the role of the liver catechol-O-methyl transferase in the metabolism of circulating catecholamines. *Acta pharmacol.* (Kbh.) *20* 47—55.

Dahlström, A. and J. Häggendal, Recovery of noradrenaline levels after reserpine compared with the life-span of amine storage granules in rat and rabbit. *J. pharm. pharmacol.* 1966 *18* 750—75.

Euler, U. S. von and F. Lishajko, Effect of drugs on the storage granules of adrenergic nerves. In *Pharmacology of Cholinergic and Adrenergic Transmission*. Ed. G. B. Koelle, W. W. Douglas and A. Carlsson. Prague 1965 245—259.

Häggendal, J. and B. E. Roos, The effect of benzquinamide on the metabolism of catecholamines in rabbit brain. *Acta pharmacol.* (Kbh.) 1967 (In press).

Hess, S., P. A. Shore and B. B. Brodie, Persistence of reserpine action after disappearance of drug from brain: effect on serotonin. *J. Pharmacol.* 1956 *118* 84—89.

Hillarp, N. A., Isolation and some biochemical properties of the catechol amine granules in the cow adrenal medulla. *Acta physiol. scand.* 1958 *43* 82—96.

Kirshner, N., Uptake of catecholamines by a particulate fraction of the adrenal medulla. *Science* 1962 *135* 107—108.

Lindmar, R. and F. M. Muscholl, Die Wirkung von Pharmaka auf die Elimination von Noradrenalin aus der Perfusionsflüssigkeit und die Noradrenalinaufnahme in das isolierte Herz. *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* 1964 *247* 469—492.

Lundborg, P., Storage function and amine levels of the adrenal medullary granules at various intervals after reserpine treatment. *Experientia* (Basel) 163 *19* 479.

Pletscher, A., A. Brossi and K. Gey, Benzoquinolizine derivatives: a new class of monoamine decreasing drugs with psychotropic action. *Int. Rev. Neurobiol.* 1962 *4* 275—306.

Quinn, G. P., P. A. Shore and B. B. Brodie, Biochemical and pharmacological studies on Ro 1-9569 (tetrabenazine), a non-indole tranquillizing agent with reserpine-like effects. *J. Pharmacol.* 1959 *127* 103—109.

Seiden, L. and A. Carlsson, Reversal of the reserpine-induced suppression of the conditioned avoidance response in the cat by L-DOPA. *Psychopharmacologia* (Berl.) 1964 *6* 239—244.

Stitzel, R. E. and P. Lundborg, Effect of reserpine and monoamine oxidase inhibition on the uptake and subcellular distribution of ^{3}H-noradrenaline. *Brit. J. Pharmacol.* 1967 *29* 99—104.

Weissman, A. and K. Finger, Effects of benzquinamide on avoidance behaviour and brain amine levels. *Biochem. Pharmacol.* 1962 *11* 871—880.

Acta physiol. scand. 1968. 72. 62—64

From the Institute of Occupational Health, Työterveyslaitos, Helsinki, Finland

Effect of Apple and Pectin Diets on Serum and Liver Cholesterol in Rats

By

ESKO KARVINEN and MATTI MIETTINEN

Received 3 May 1967

Abstract

KARVINEN, E. and M. MIETTINEN. *Effect of apple and pectin diets on serum and liver cholesterol in rats.* Acta physiol. scand. 1968. 72. 62—64.

Male rats were fed isocaloric diets in which were incorporated 46.4 g unpeeled apple or 2 g pectin per day per rat. Serum cholesterol level of the rats on the apple diet was significantly lower than that of the controls. Serum cholest rol level of the rats on th pectin diet was not altered significantly. The amounts of liver cholesterol were not significantly hanged by the apple or pecti diet. Uptake of orally given cholesterol-4-C^{14} in the liver unsaponifiable lipid was lower in the pectin fed and pple fed animals compared with that of the controls.

It has been reported (Lin *et al.* 1957) that in rats the addition of pectin to a basal diet containing cholesterol increased the excretion of fecal saponifiable and nonsaponifiable lipids and decreased the absorption of cholesterol. It has also been reported by Keyes *et al.* (1961) that in man the addition of 15 g of pectin to the y diet produced a 5 per cent decrease in the serum cholesterol level. It was considered worthwhile to study further these effects of pectin on the cholesterol metabolism.

Material and Methods

Male rat of he Wistar stra weighing 250—300 g were caged individually and fed a experimental basal diet for 1 week. The basal diet onsisted of the following: 11 g c sein, 6.78 g sucrose, 0.68 g yeast and 0.43 g salt mixture. I each case 10 g of the basal diet g daily supplemented with 1 g of corn oil per day. After one week on the basal diet the rats were divided into groups which were given one of the following experimental diets in isocaloric ration. (1) The *pectin diet* consisted of the following amount per rat per day: 11 g casein, 4.78 g sucrose, 0.68 g yeast, 0.43 g salt mixture and 2.00 g pectin, supplemented with 1 g of corn oil. (The *pple diet* consisted of the following amounts pe t per day: 11 g casein, 0.68 g yeast, 0.43 g salt mixt re and 46.4 g peeled pple supplemented with 1 g of corn oil. (3) The *r ol diet* was the basal diet given abov supplemented with 1 g of corn oil.

After 4 weeks on the experimental diet each rat wa made t f st overnight and fed 3 µc of cholesterol-4-C^{14} in the daily experimental ration he following morning 9.00. Th labeled cholesterol was supplied by the Radiochemical Centre, Amersham, England. Cholesterol-4-C^{14} was dissolved in ether nd poured on the dry basal diet. Than the dry diet w made

into paste by adding water. The rats ate the paste in the usual manner. On the following 6 days the rats were fed the same experimental rations that they had been consuming earlier during the experiment.

Six days after feeding the labeled cholesterol to the rats, they were anesthetized with ether and bled from the tail, and then the livers were excised and immersed in ethanol. Serum cholesterol was determined by the method of Pearson et al. (1953). The amount of unsaponifiable lipid of the liver and the radioactivity of the liver lipid were determined as has been described previously (Karvinen et al. 1964).

Results

Serum cholesterol. Serum cholesterol values of the pectin, apple and control groups are given in Table I. Serum cholesterol levels in the pectin fed animals did not significantly differ from those of the controls on the basal diet. On the other hand, the serum cholesterol level of the rats on the apple diet were significantly lower than that of the controls.

Liver cholesterol. The effects of pectin and apple diets on rat liver cholesterol and on the uptake of labeled cholesterol in the liver are given in Table II.

There was no significant difference in the amount of liver cholesterol between the pectin-fed and control animals, as well as between the apple-fed animals and their controls.

Uptake of cholesterol-4-C^{14} in the liver unsaponifiable lipid was lower in the pectin-fed animals than in the control groups, the difference being significant in pectin group II and insignificant in pectin group I. Also in the apple fed animals, incorporation of labeled cholesterol in the liver unsaponifiable material was significantly lower than in the control group.

Discussion

The present results show that feeding an apple diet to rats leads to a reduction of the serum cholesterol level, whereas feeding them a pectin diet does not produce any significant change in this serum cholesterol level. The literature does not contain data on the effect of pectin or an apple diet on the serum cholesterol level in the rat. Erschoff (1963) however reports that pectin N.F. when fed at levels of 10 per cent or higher in the diet, prevented experimentally induced atherosclerosis

TABLE I. Effect of pectin and apple diets on rat serum cholesterol.

Group	Number of rats	Serum cholesterol mg%	P
Pectin I	8	51.1 ± 8.4	non significant
Basal diet I	8	58.3 ± 9.3	
Pectin II	9	58.0 ± 8.4	non significant
Basal diet II	10	62.7 ± 10.2	
Apple III	10	49.2 ± 5.5	0.01
Basal diet III	10	57.1 ± 8.1	

TABLE II Effect of pecti nd pple diets on rat liver cholesterol and on the uptake of ingested cholesterol-4 C[14] in liver lipid

Group	Number of rats	Liver cholesterol mg	P	Dose of cholesterol-4-C[14] uc	Total ctivity of liver unsaponifiable lipid cp 100 sec	P
Pectin I	6	59 7±12.7	non significant	5	8540±1241	non significant
Basal diet I	6	41.8±11 7		5	12522±5745	
Pectin II	10	51 1±9 7	non significant	1	1196±292	0.01
Basal diet II	10	59 1±13.8		1	1890±714	
Appl III	10	61 4±15 6	on significant	5	11505±3238	0.02
Basal diet III	10	65.8±10 7		5	14845±2171	

in the rat. On the basis of Erschoff's study pectin must be fed at levels of 10 per cent or higher in the diet for an optimal protective effect.

In man, on the other hand, the addition of 15 g of pectin to the daily diet produced a 5 per cent decrease in the serum cholesterol level (Keyes *et al.* 1961) Our results do not necessarily contradict those which concern man the means for serum in both pectin experiments (Table I) are lower than those of the control experiments even if the differences are not ignificant

The rats on the apple diet developed rather loose stools during the experiment. The ga trointestinal passage-time was evidently shortened. This might have affected the absorption of holesterol and therefore might have been reflected in the serum cholesterol level.

The present results show no significant difference in the liver cholesterol level between the pectin and control animals. However the incorporation of oral cholesterol-4-C m the li er unsaponifiable lipid was les in the pectin fed and apple diet groups than in the controls In the apple diet, this was apparently due to a reduced absorption of cholesterol from the intestine In the pectin diet this result might pos bl ha lso been due to a reduced intestinal absorption of cholesterol

References

KARVINE E O K and M MIETTIN Effect of ethionine on the incorporation of palmitic acid 1-C in ra serum lipoproteins *A t physiol scand* 1964 62 150—155
ERSCHOFF B H Effec of pectin N F and other complex rbohydrates on liver holesterolemia nd herosclerosis *F p M d S g* 1963 21 108—11
KEYS A, F GRA DE nd J T A DERSON Fiber and pectin in the diet and serum holesterol concen ra ion in man *P oc Soc p Biol (N Y)* 1961 106 555—558
LIN T M K S KIM E KAR nd A C IVY Effect of dietary pectin, protopectin and gum arabic on holesterol excretion in ra *Amer J Physiol* 1957 188 66—70
PEARSON S S STE and T H MACGOVACK, A rapid accurat method for the determination of total holesterol in serum *Anal Ch m* 1953 25 813—814

Acta physiol. scand. 1968. 72. 65—71

From the Research Institute of National Defence Dept 1 Stockholm, Sweden

Differences in the Subcellular Localization of Choline, Acetylcholine and Atropine Taken up by Mouse Brain Slices in vitro

By

J SCHUBERTH and A. SUNDWALL

Received 3 May 1967

Abstract

SCHUBERTH, J and A. SUNDWALL. *Differences in the subcellular localization of choline acetylcholine and atropine taken up by mouse brain slices in vitro.* Acta physiol. scand. 1968. 72 65—71

Mouse brain cortex slices have been incubated with tritium labelled acetylcholine choline and atropine. The subcellular distribution of radioactivity has then been studied by homogenization of the slices in sucrose and separation of the subcellular particles on continuous exponential sucrose gradient. It was found that abou 30 per cent of the radioactivity in the slices were localized in the fraction containing nerv -endings and mitochondria. Using differential centrifugation and gradient centrifugation of the crude mitochondrial fraction obtained, it was found that acetylcholine is specifically bound to the nerv -ending particles. Choline had sub-cellular distribution similar to acetylcholine, while tropine was not specifically bound to the nerve-endings.

It has recently been shown that choline (Schuberth *et al.* 1966) and acetylcholine (AcCh) (Schuberth and Sundwall 1967 a) are taken up by brain cortex slices by an active transport mechanism. *I vivo* the major part of the acetylcholine in the brain is localized in the nerve-endings (Hebb and Whittaker 1958) It is in these structures that the acetylation of choline to AcCh is also assumed to occur since most of the choline acetyltransferase ctivity is confined to the nerve-endings (Hebb and Whittaker 1958) The experiments which will be described in this communication were undertaken in order to investigat a possible specific localization to the nerve-endings of choline and AcCh taken up by brain cortex slices.

Kinetic data indicate that cholinergic blocking agents such as atropine combine with structures in the postsynaptic membrane Since pinched-off nerve-endings also include the postsynaptic membrane (Gray and Whittaker 1962) the subcellular distribution of a cholinergic blocking drug has been tudied. Atropine has been used since it is also taken up by brain cortex slices against a concentration gradient (Schubert and Sundwall 1967 b)

Experimental

Mouse brain cortex slices were incubated in modified Krebs-Henseleit solution (Schuberth et al. 1966). When ACh was studied 10^{-6} M Sarin was used to inhibit cholinesterase. After 15 min preincubation at 37 C, the tritium labelled drug under study was introduced by tipping from the side arm of the Warburg flask. The slices were incubated for 60 min and then removed from the vessels, rinsed in 10 ml fresh incubation medium (without Sarin) and homogenized in 0.32 M sucrose (10 per cent tissue using a Potter-Elvehjem all-glass homogenizer) under carefully controlled conditions at +3 C.

Fractionation of subcellular particles by sucrose gradient centrifugation. A continuous exponential sucrose gradient was prepared with a constant volume mixer of 5 ml volume (Peter and Axelrod 1962). The mixer was filled with 1.73 M sucrose and fitted with magnetic stirrer whereupon 0.32 M sucrose was added from a reservoir through fine glass tip near the bottom of the mixer. The outflow from the mixer was collected in an ultracentrifuge tube. The shape and reproducibility of the gradient was checked by adding picric acid to the mixer and measuring the absorbancy at 354 mμ in 0.2 ml portions.

0.5 ml of the homogenate was carefully layered on the top of 4.5 ml sucrose gradient in centrifuge tubes and centrifuged for 60 min at 36,000 rpm in the Spinco model L ultracentrifuge (head SW 39 L). After centrifugation, photographs of all centrifuge tubes were taken with polaroid camera in order to control the reproducibility of the positions of the bands. Consecutive 0.2 ml portions of the gradient were taken for radioassay by liquid scintillation and 1 ml portions for determination of cytochrome oxidase. The fractions were collected by means of an infusion pump with syringe containing chloroform connected to the bottom of the centrifuge tube via polyethylene tube and needle. To facilitate collection of the fractions the centrifuge tube was fitted with stopper and U-shaped glass tube. In order to minimize turbulence the stopper had conic excavation.

Fractionation of subcellular particles by differential centrifugation and discontinuous gradient. 5 ml of the homogenate was centrifuged (Sorvall, head SS 34) at 3,000 rpm for 10 min (1,000 × g) in order to remove nuclei and cell debris. The supernatant was then centrifuged at 13,000 rpm for 15 min (20,000 × g). The pellet which mainly consists of mitochondria and nerve-ending particles was suspended in 1 ml 0.32 M sucrose and carefully layered on the top of discontinuous gradient consisting of 2 ml 0.8 M and 2 ml 1.2 M sucrose. The sample was then centrifuged in Spinco model L ultracentrifuge (head SW 39 L) at 39,000 rpm for 1 hr (120 000 × g). As described in the original reference (Whittaker 1959) two bands and a pellet were obtained. The upper bands contain myelin, the lower nerve-endings and the pellet mitochondria. The bands and the clear solution between were carefully removed with pasteur pipett and the pellet suspended in 1 ml 0.32 M sucrose. Aliquots were assayed for radioactivity and choline acetyltransferase activity.

Determination of choline acetyltransferase activity. 0.25 ml of the fractions were activated with 0.1 ml ether (Hebb and Smallman 1956). The enzyme activity was determined according to the method of McCaman and Hunt (1965). The following modifications were introduced in the original procedure. Tritium labelled acetyl-CoA of five times higher specific activity (50 mC/mmol) was used and the reineckate ion and acetyl-CoA removed on an ion exchange resin prior to radioassay (Schuberth and Sparf 1967).

Determination of cytochrome oxidase activity. The fractions were incubated at 37 C in total volume of 3 ml. The incubation medium consisted of 2 mg cytochrome C, 0.5 ml 0.5 M phosphate buffer pH 7.5 and 1.0 ml of the test sample. The reaction was started by addition of 15 μl 1 M ascorbic acid and 10 μl of fresh solution of 7.1 mg tetramethyl-p-phenylenediamine dihydrochloride in 1 ml 0.5 M tris buffer pH 7.5. The oxygen consumption was measured with polarographic electrode (Beckman Model 160 Physiological Gas Analyzer) under liquid paraffin and continuous stirring (G. Dallner personal communication).

Radioassays were performed by liquid scintillation in Nuclear Chicago Model 720 liquid scintillation counter. To 1 ml of sample was added 14 ml of scintillation solution containing 960 ml dioxane, 5.12 g PPO, 0.128 g POPOP and 102.4 g naphtalene. Quench corrections were made by the channels ratio procedure. This was justified since high (20 per cent) and reproducible counting efficiency was obtained.

Electron microscopy. After dilution with 0.32 M sucrose the fractions supposed to contain myelin, nerve-endings and mitochondria respectively were spun down at 120 000 × g for 60 min. The pellets were removed with spatula and fixed in osmium tetroxide. Several pieces through the entire thickness of the pellet were embedded in Epon 812 and sectioned. The sections were stained with saturated lead acetate and saturated uranyl acetate and examined under an Elmiskop I A Siemens electron microscope.

Results

Distribution of radioactivity in continuous gradient

As seen in Fig 1 about 30 per cent of the total radioactivity of the slices which had been incubated with tritium labelled AcCh (2.0×10^{-8} M) are confined to the fractions containing nerve-ending particles and mitochondria. In this experiment the whole homogenate was centrifuged on a continuous exponential sucrose gradient. Since the peak of radioactivity coincided with that of cytochrome oxidase activity it was assumed that the AcCh is bound to mitochondria and/or nerve-ending particles. When the slices were incubated with radioactive choline (2.0×10^{-8} M) a similar subcellular distribution was found. However following incubation with radioactive atropine ($2.0 \times 10^{-}$ M) no accumulation of radioactivity occurred.

Distribution of radioactivity in myelin nerve-endings and mitochondria

In order to investigate whether the drugs are specifically bound to mitochondria and/or nerve-ending particles, the crude mitochondrial fraction obtained after differential centrifugation was separated on the discontinuous sucrose gradient. As seen

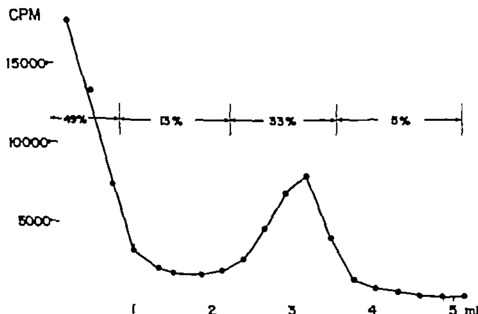


Fig 1 Subcellular distribution of tritium labelled acetylcholine taken up by mouse brain cortex slices. The slices were incubated in medium containing 2×10^{-8} M tritium labelled AcCh, washed, homogenized and centrifuged on continuous sucrose gradient (see

ChAc activity[1]		CPM per 0 2 ml sampl		
		a tylcholine	choline	tropine
13± 7 6		2365±212, 0	472± 77 0	1322±234, 0
9± 4, 1		708± 21 4	314± 26, 6	544±110, 0
6± 3, 9		361± 34, 5	309± 66, 3	213± 64, 2
166±40, 4		1101±236, 0	917±234, 0	163± 53, 8
16± 7 7		304± 39 8	217± 28, 7	73± 16, 4
20± 6, 8		625±162, 0	837±266, 0	129± 23, 2

Fig. 2. Distribution of radioactivity and holine acetyltransferase activity (ChAc[1]) in the crude mitochondrial fraction of mouse brain cortex slices following incubation with tritium labelled acetylcholine choline and atropine The slices were incubated in medium containing 2×10^{-8} M tritium labelled compound (2—4 μC) The crude mitochondrial fraction was centrifuged on a discontinuous sucrose gradient (see text) Radioactivity and ChAc-activity were assayed in the different layers of the gradient

ChAc-activ ty was assayed in 0 2 ml fractions and expressed as mμ moles AcCh formed per 30 min and 0.2 ml sample

in Fig 2 following incubation of the slices with tritium labelled AcCh or choline ·oactivity was bound both to the fractions containing pinched-off nerve-endings d mitochondria After incubation with atropine on the other hand no specific ccumulation of radioactivity is found in the nerve-ending fraction (Fig 2)

Id nt ficat on of the d fferent subcellular fractions

The efficiency and reproducibility of the separation technique was controlled by choline acetyltransferase determinations and by electron microscopy As seen in Fig 2, about 72 per cent of the total enzyme activity is present in the "nerve-ending fraction" Electron microscopy of the isolated fractions reveal that very few mitochondria contaminate the nerve-ending fraction (Fig 3) The membrane fragments seen are probably derived from nerve-endings disrupted d ring fixation.

Non specific b d g of radioactivity duri g hom genisation

In order to in te the possibility that the accumulation of AcCh and choline in the nerve-end ti tion is the result of non-specific binding during homogenisation, cortex slic w homogenized in sucrose containing tritium labelled AcCh and choline A sec t He I AcCh and choline are not bound to sub-cellular particles during or af l genization.



Fig. 3 Electronmicrograph of a nerve-ending fraction (12,000 ×). Some nerve-endings are indicated by arrows. The membrane fragments seen are presumably derived from nerve-endings disrupted during fixation. No free mitochondria can be detected.

Localization of radioactivity in the nerve-ending

Following incubation of the slices with labelled AcCh the nerve-ending fraction was isolated and divided in two equal parts. One part was treated with distilled water in order to disrupt the membrane (De Robertis *et al.* 1963; Whittaker *et al.* 1964) and the other part was suspended in 0.32 M sucrose. The two samples were then centrifuged at 120,000 × *g* for 60 min and the supernatants assayed for radioactivity. The results from two experiments reveal that the supernatants from the disrupted nerve-endings contained 6 times the radioactivity of that of the control. The experiments thus indicate that the radioactivity is localized within the nerve-endings.

TABLE 1 Distribution of radioactivity in the crude mitochondrial fraction of mouse brain cortex slices following homogenization in 0.32 M sucrose containing tritium labelled AcCh and choline (cf. Fig. …)

Fraction	CPM per 0.2 ml sample acetylcholine	choline
1 supernatant	1 101	315
myelin	228	85
3	63	59
4 nerve-endings	50	60
5	4	18
mitochondria	52	59

Binding of radioactivity to mitochondria

Although the mitochondrial fraction contains very little nerve-endings as judged by choline acetyltransferase activity both AcCh and choline and to a certain degree, atropine are found also in this fraction.

Discussion

The results presented in this paper indicate that the nerve-endings of mouse brain cortex slices might be specifically involved in the binding of exogenous choline and acetylcholine. They also indicate that the radioactivity in the nerve-ending fraction is localized within the nerve-endings and not bound to the membrane fragments present. One major objection which could be raised against the interpretation of the results would be that the apparent specificity is merely due to an inclusion of radioactivity within the undisrupted nerve-endings while the radioactivity in the rest of the cell cytoplasm is released into the sucrose during homogenization. Such a mechanism could explain why most of the radioactivity upon centrifugation is found in the supernatant and the rest in the fraction containing cytoplasm surrounded by membranes. However that this is probably not the case is indicated by the results obtained following incubation with atropine. This drug is also taken up by brain cortex slices against a concentration gradient (Schuberth and Sundwall 1967 b) but is not accumulated in the nerve-ending fraction.

Previous studies have shown that AcCh is not metabolized in the slices under the conditions used (Schuberth and Sundwall 1967 a). Thus the radioactivity measured is almost exclusively due to unchanged AcCh. On the other hand about 50 per cent of the choline taken up by the slices are metabolized (Schuberth *et al.* 1965). Due to the relatively low specific activity of the choline used, it was not possible to conclude whether it was acetylated to AcCh in the nerve-endings or not.

It now appears to be well established that noradrenaline is, at least partly, inactivated by reabsorption from the synaptic cleft through the presynaptic membrane (Carlsson *et al.* 1965). A similar mechanism might also operate following release of AcCh into the synaptic cleft resulting in an uptake of both AcCh and the choline formed by the hydrolysis of AcCh. Actually, experiments with the perfused cervical ganglion have indicated that choline is reabsorbed by the presynaptic membrane and used for resynthesis of AcCh (Perry 1953). On the other hand Perry concluded that AcCh was not reabsorbed. However, this may be due to the presence of eserine which has recently been shown to inhibit the uptake of AcCh in brain cortex slices (Polak and Meeuws 1966, Schuberth and Sundwall 196 a).

Since nerve-ending particles isolated from sucrose homogenates include the postsynaptic membrane (Gray and Whittaker 196), it might have been expected that atropine should have accumulated in the nerve-ending fraction. On the other hand

the binding of atropine to the cholinergic receptor probably being extracellular might be too loose to be detected.

The authors are indebted to Mrs K. Ekblad and Mrs B. Lobergh for skilful technical assistance and to Miss I. Sjödén for the electron microscopy.

References

CARLSSON, A., A. DAHLSTRÖM, K. FUXE and M. LINDQVIST: Histochemical and biochemical detection of monoamine release from brain neurons. *Life Sci.* 1965. *4*. 809—816.

DALLNER, G., Pers. comm.

GRAY, E. G. and V. P. WHITTAKER, The isolation of nerve-endings from brain: an electron microscopic study of cell fragments derived by homogenization and centrifugation. *J. Anat.* (Lond.) 1962. *96*. 79—88.

HEBB, C. O. and B. N. SMALLMAN, Intracellular distribution of choline acetylase. *J. Physiol.* (Lond.) 1956. *134*. 385—392.

HEBB, C. O. and V. P. WHITTAKER, Intracellular distribution of acetylcholine and choline acetylase. *J. Physiol.* (Lond.) 1958. *142*. 187—196.

MCCAMAN, R. E. and J. M. HUNT: Microdetermination of choline acetylase in nervous tissue. *J. Neurochem.* 1965. *12*. 253—259.

PERRY, W. L. M.: Acetylcholine release in the cat's superior cervical ganglion. *J. Physiol.* (Lond.) 1953. *119*. 439—454.

POLAK, R. L. and M. M. MEEUWS, The influence of atropine on the release and uptake of acetylcholine by the isolated cerebral cortex of the rat. *Biochem. Pharmacol.* 1966. *15*. 989—992.

POTTER, L. T. and J. AXELROD, Intracellular localization of catecholamines in tissues of the rat. *Nature* (Lond.) 1962. *194*. 581—582.

SCHUBERTH, J., A. SUNDWALL, B. SÖRBO and J.-O. LINDELL, Uptake of choline by mouse brain slices. *J. Neurochem.* 1966. *13*. 347—352.

SCHUBERTH, J. and A. SUNDWALL, Effects of some drugs on the uptake of acetylcholine in mouse brain cortex slices. *J. Neurochem.* 1967. *14*. 807—812.

SCHUBERTH, J. and A. SUNDWALL, To be publ.

SCHUBERTH, J. and B. SPARF, To be publ.

WHITTAKER, V. P.: The isolation and characterization of acetylcholine-containing particles from brain. *Biochem. J.* 1959. *72*. 694—706.

Acta Physiol. scand. 1968. 72. 72—80

From the Departments of Aviation and Naval Medicine, Faculty of Medicine, Karolinska Institutet, Stockholm, Sweden

Antigravity Effects of Leg Exercise

By

G. Rosenhamer

Received 8 May 1967

Abstract

Rosenhamer, G. *Antigravity effects of leg exercise.* Acta physiol. scand. 1968. 72. 72—80.

To investigate the effects of leg exercise on the overall tolerance to exaggerated gravitational stress, 8 subjects were exposed for 15 min to a threefold increase of the force of gravity in centrifuge experiments (+ 3 G) while in the sitting position, both during motionless resting and when exercising at 600 kpm/min on a bicycle ergometer. In all subjects, continuous leg exercise prevented the occurrence of circulatory collapse which otherwise became imminent in 5 subjects after 5 to 12 min in the resting condition as evidenced by blackout or rapid increase in heart rate. Subjectively, exercise was experienced as less stressful than the resting condition. The improved circulatory stability with exercise was also reflected by uniformity of individual heart rate responses, the group mean value increasing from 152 to 160 beats/min during the 6th to 12th min.

Cardiovascular tolerance in man to motionless standing is limited and it is well known that the force of gravity eventually acts to bring about a progressive fall of the systemic arterial pressure at head level followed by circulatory collapse (for review see Gauer and Thron 1965). Border-line tolerance is reached more rapidly when the acting force is magnified through use of the human centrifuge. Thus, exposure of unprotected individuals to a 4 to 7-fold increase of the force of normal gravity in the sitting position generally causes loss of vision or consciousness within 4—6 sec because of insufficient perfusion pressure at eye or head level (Wood *et al.* 1946, Henry *et al.* 1951). These consequences of gravity and accelerative forces acting in the head-to-foot direction are largely due to increased hydrostatic pressure differences in the vascular tree. Leg exercise in the erect position, as in walking or sitting as in bicycling, *increases the systemic arterial pressure at normal gravity* and so exerts a protective action on orthostatic tolerance.

So far no investigations have been carried out as to the effects of graded leg exercise on the cardiovascular tolerance to forces stronger than that exerted by normal gravity. Such experiments seem to be of considerable interest since the

TABLE I Individual data

Subj. no.	Age, years	Weight, kg	Height, cm	Heart rate, beats/min			PWC$_{170}$ kpm/min
				lying at rest	standing at rest after 8 min	6th min of exercise at 600 kpm/min	
1	20	70	181	55	74	128	1070
2	24	68	185	68	94	105	1550
3	23	50	173	81	107	142	850
4	21	74	186	62	102	125	1020
5	22	63	172	60	75	117	1090
6	24	78	191	55	92	109	1560
7	24	58	162	63	79	127	1040
8	26	72	185	59	95	115	1230

All functional data obtained at normal gravity
PWC$_{170}$ = physical working capacity at pulse rate 170.
(Sjöstrand 1947, Wahlund 1948) sitting position (Fig. 1)

normally protective effect of leg exercise on orthostatic tolerance might theoretically be cancelled by the greater hydrostatic pressure that develops in the dependent leg muscles during increased gravitational stress. In the present study the effects of leg exercise in the sitting position on the electrocardiographic, heart rate and subjective responses to a simulated increase of gravity to three times its normal value were investigated.

Subjects, Methods and Procedure

Subjects Experiments were performed on 8 healthy male students. All were familiar with the subjective sensations experienced during runs in the centrifuge. Heart rates immediately prior to the runs were not higher than those normally observed at rest in the sitting position, and no one showed other signs of apprehension or anxiety prior to or during the runs. Individual dimensional and functional data are given in Table I. Physical working capacities were average, except in one subject (no. 3).

Methods All subjects were studied while seated in the centrifuge cabin suspended 7.3 m from the center of rotation (for description of the centrifuge, see Gotzlinger and Helburg 1955, Bjurstedt 1957). Throughout all runs the subject leant against a backrest inclined 13° from the vertical and had his occiput in contact with a headrest. His feet were fastened on the pedals of an electrodynamically braked bicycle ergometer which was modified so that the axis of the pedals was at the level of the seat. On starting the centrifuge the cabin swings out freely so that the resultant of the gravitational and centrifugal G vectors remains aligned with the head-seat axis (sagittal plane) of the body. The final magnitude of the resultant G vector was preset to 3 G units; the resultant G profile as a function of time had the shape of a single plateau. In this manner the subject could thus be exposed to a force three times greater than that exerted by normal gravity. Fig. 1 shows the magnitude and direction of G in relation to body geometry prior to and during the centrifuge runs. G-suits were not used.

Ergometer An electrically braked bicycle ergometer (Holmgren and Mattsson 1954) was used in which the work load could be preset and was largely independent of pedal rpm.

Synonyms often used to indicate the magnitude and direction of the force employed: positive or headward acceleration, + 3 G.

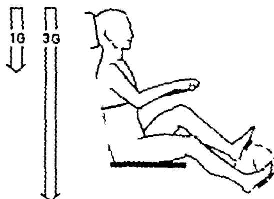


Fig. 1 G-forces and body contour with the subject seated in the centrifuge cabin left arrow indicates the direction and magnitude of the force with the centrifuge standing still, right arrow with the centrifuge running.

The increase of power dissipation in the ergometer resulting from the increased G-force was estimated by driving the generator of the ergometer as a motor at rate of 350 rpm (*i.e.* the speed of the generator vis in work tests at the tandard pedal rate of 60 rpm) and measuring the energy expenditure of the motor at increasing G-levels. The increase in expenditure measured in this way was found not to exceed 5 kpm/min at 3 G

Elect ocardiogr ms were recorded with a direct writing recorde (Mingograph 42, Elema, Stockholm) t paper speed of 50 mm/sec Leads I, II III aVR, VL, aVF CR 1 4-5-7 were recorded before the centrifuge runs, with the subject in recumbent and tanding (after 8 mi) positions, respectively and then while sitting in the cabin. These leads ere also recorded for 3 min following the centrif ge runs. During the exposures to 3 G leads CH -4 5-7 (indifferent lectrode on the forehead, *cf* Holmgren and Strandell 1961) ere recorded for the la 15—20 sec of each min, whether the subject was working or not

The lectrocardiograms were examined nd lassified according to Grewin (1948) and Strandell (1963 ST-depressions were measured from horizontal line through the end of the preceding P R segment. The lower border of the recorded lines was used as reference for the measurements

Heart at w recorded on photographic paper by means of an instantaneous cardiotachometer (Sturm and Wood 1917)

Blood lact ac d was determined in arterialized' finger blood within 2 mi of cessation of G exposures by the colorimetric method of Barker and Summerson (1941) as modified by Strom 194

Physical ork g pacity At normal gra ity the pproximately linear relationship between heart and ork load as used to obtain, by extra or intrapolation, the rate of ork in kpm/min corresponding to heart rate of 170 beats/min (PWC 170 Sjöstrand 1947 Wahlund 1949

Orth static Heart t and ECG ere recorded after 8 min of motionless standing against all

Proc d All subjects wer exposed t 3 G tw entrif ge runs, each of 13 min duration. Throughout one ru the subject rested with his fee supported by the ergometer pedals, and w instructed relax and to remai ompletely motionless In the other he rested during the first min and hen exercised onst nt load of 600 kpm/min for 12 min. until the end of the ru The ra of pedalling was 60 rpm Both runs ere preceded by t least 60 min rest t 1 G The sequenc bet een the 3 G rest nd 3 G exerc runs as rotated among the subject Whereas the heart te and ECG wer recorded ontinuously during both types of runs blood samples for lact acid determination er onl taken immedia l before and after the runs.

A centrif ge run ould rapidl be terminated in he event of signs of intoleranc or if so ev the subject desired For this purpose ore ommunic tion between the subject and the investigator was possible all imes I addi ion, he subjec ondition was hecked by constant surveillanc of the subject face and pper part of his torso by losed-circuit television, and by monitoring udible he rt rat and lec rocardiographic tracings The temperature in the entrif ge als red between 23 and 25 C during both rest nd exercise

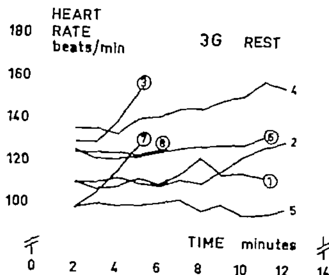
Results

Subjective actions The subjective sensations associated with the 3 G rest experiments were generally relatively stressful. This was due only to a minor degree to the increased weight of different body parts, and more to a progressively increasing feeling of fatigue and, in six of the eight subjects, dimming of peripheral or central vision following the first 4 to 5 min. In 5 cases, rapidly increasing dimming of vision and impending loss of consciousness necessitated a shortening of the 3 G rest exposures. In three of these cases the runs were discontinued during the 6th to 7th min, and in two cases during the 12th min (see Fig 2)

By contrast, when leg work was performed for the last 12 of the 13 min at 3 G no case of dimmed vision occurred, and no run had to be shortened. 3 G exercise was experienced by all subjects as less exhausting and unpleasant than 3 G rest. Moving the pedals was not subjectively experienced as more difficult at 3 G than at normal G One type of discomfort, however was more pronounced during exercise, rz a dull, aching sensation in the neck which increased moderately during the course of the run. Movement of the head did little to relieve the pain. There was no pain in the lower back or lower extremities, and in none of the experiments was substernal pain reported.

El ct o-c diographic findings Control ECG.s at rest and at normal G n the sitting position were all normal. In subject no. 4 the T-waves in leads II, III CR5 and CR7 were slightly diphasic or isoelectric during the orthostatic test. The changes in this case were classified with Strandell (1964) as moderate but not marked. No ectopic beat or other marked alterations in the ECG.s were observed either at 3 G rest or 3 G exercise Thus, in no case during or after work did ST junction depressions exceed 0.5 mm, or changes to horizontal or downward sloping ST-segments ppea No marked flattening of the T-waves was observed. According to Strandell's nomenclature no ECG during exercise was classified as intermediate or suspected abnormal" The subject who had shown moderate changes in the orthostatic test displa ed no such changes during the centrifug runs.

H t t Individual heart rates during 3 G rest and exercise, averaged over 1-min period are shown in Fig 2. In the rest experiments at 3 G all subjects showed highe hea t rates fter 2 min than after 8 min motionless standing at normal G (/ T ble I The range of individual responses at 3 G rest was rather wide, how ever Thus 2 subjects (no 3 and 7) showed rapidly increasing heart rates over the last to 3 min of the run before it had to be terminated in the 6th min because of mpending unconsciousness. In two other cases (no 6 and 8) where the exposures had t be terminated for the same reason, the heart rate showed a rapid increase only du ng the last few seconds, as observed on the original photokymographic re dings. There seemed to be no correlation between th incidence of blackout nd the increments in heart rate observed after 8 min standing at normal G or after to 4 min exposure to 3 G rest. During the course of the 3 G exercise runs, a



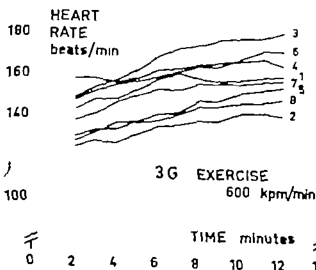


Fig. 2. Heart rates (individual averages for consecutive minutes of observation) 1–8 subjects in the sitting position at 3 G rest (upper graph) and at 3 G exercise at 600 kpm/min (lower graph) with individual responses numbered for identification. Values obtained during the first 2 min have been ommitted. Circles indicate elective termination of experiment because of impending loss of consciousness.

progressive increase of the heart rate occurred in all cases. The mean value of individual time averages over the 6th min of exercise was 152 beats/min, and had increased by 4 beats by the 9th min (range 1 to 12) and by another 4 beats (range 2 to 14) by the 12th min.

Blood lactates. Individual values are shown in Table II. Exposure to 3 G rest for 15 min resulted in no systemic change of the resting control values obtained at normal G. With 600 kpm/min for the last 12 min at 3 G the average lactate con-

TABLE II. Arterial lactate concentrations (mM/l) obtained at 1 G rest (control) and within 2 min following 3 G rest and 3 G exercise

Subject No.	1 G rest	3 G rest	3 G 600 kpm/min
1	77	.60	1 40
2	78	79	3.13
3	1.00	1.62	3.48
4	1 08	—	1.80
5	1 40	.93	2.03
6	1 07	.63	1.90
7	.65	1 15	2.28
8	75	.93	3 45
Mean	.94	.95	2.96
SE	.09	60	.51

Highest value based on duplicate determinations on each of two consecutive blood samples.

centration attained a value of 2.56 meq/l, as compared to 0.95 meq/l for 3 G rest No individual value at 3 G exercise exceeded 3.5 meq/l. There seemed to be no correlation between the heart rate and lactate concentration during exercise.

Discussion

Leg exercise and G tolerance In the experiments with exposure to 3 G during motionless resting, the occurrence of blackout with impending unconsciousness necessitated termination of the centrifuge runs in 5 of the 8 subjects before the preset time period of 13 min had elapsed. By contrast, exposure to the same G level was experienced as less stressful when combined with leg exercise, and vision and consciousness were well maintained in all subjects throughout the runs. These are clear indications that leg exercise involves physiological adjustments favourable to G tolerance Loss of central and peripheral vision during headward acceleration, which precedes loss of consciousness, and which may be used as an index of G tolerance (Gauer and Zuidema 1961) is generally attributed to a reduction of the arterial perfusion pressure at eye level to values inadequate to overcome intraocular pressure (Lambert 1945) The absence of visual impairment suggests that exercise elevated the arterial pressure sufficiently to maintain an effective blood supply to the eyes and brain.

With headward acceleration, the increased effective weight of blood causes the arterial pressure to fall in the upper portion of the body and to rise in the dependent regions (Wood et al 1946) The pressure reduction at head level is partly due to the increased hydrostatic pressure difference between the head and heart, and partly to a curtailment of the cardiac output secondary to sequestration of blood in

distensible veins in dependent regions of the body In this situation, reflex constriction of resistance vessels in these regions would counteract downward shunting of arterial flow and tend to improve the supply of blood to the brain (Brown, Wood and Lambert 1949 cf Gauer and Thron 1965)

With leg exercise, local blood flow must increase to preserve an adequate oxygen supply to the working muscles. The observation that no impairment in vision and consciousness appeared at 3 G when combined with exercise indicates that the total cardiac output was sufficiently increased not only to permit an increase of the blood flow through the dependent legs, but also to maintain an adequate blood supply to the eyes and the brain The observed improvement of the circulation at head level may also to some extent have been due to compensatory exercise induced arterial vasoconstriction in body regions which were not actively engaged in the work performed, such as occurs at normal gravity (see Rushmer 1961) Thus, a more forceful vasoconstriction during exercise, e g., in the splanchnic region may have been superimposed upon the initial vasoconstriction induced by acceleration

Heart rate The increase in the resting heart rate during exposure to 3 G was of the same order of magnitude as observed in previous investigations by Bjurstedt Hansson and Ström (1959) and by Wood et al (1961) Presumably the response of the heart rate was compensatory for a reduced stroke volume secondary to impaired cardiac filling (cf Wood et al 1961) While the initial compensatory increase of the heart rate generally levelled off after the first 2 min, a rapid secondary rise occurred in 4 of the 5 cases in which blackout or unconsciousness was imminent. This response pattern was evidently indicative of impending failure of compensatory adjustments

By contrast, the observed further elevation of the heart rate during exercise (Fig 2) must have been combined with partial restoration of cardiac filling, leading to improvement of the stroke volume and cardiac output. Enhancement of cardiac filling would in turn result from the repetitive action of the leg muscle pump as long as pooling of blood in distensible intra-abdominal vessels is counteracted, e g by adequate external support from the abdominal muscle wall.

ECG reactio The absence of abnormal ECG patterns during exercise at high G is of particular interest in view of the debated question whether increased gravitational stress may in olve risks for coronary insufficiency (cf Gauer and Zuidema 1961 Sieker 1961) That no sign of functional disturbances were revealed under resting conditions is in accordance with observations by Bjurstedt Hansson and Ström (1959) The present results indicate that any potential coronary insufficiency at 3 G was in any case not aggravated by moderate exercise

Oxygen transport Though a rise in the cardiac output implied an increment in the oxygen transport capacity of the circulation it may be questioned whether the resulting oxygen supply to the working muscles was adequate in relation to the

metabolic need. For instance one might expect that G-induced vasoconstriction in the leg muscles interfered with the oxygen supply during exercise. However the observed blood lactate concentrations indicated that such interference must have been slight. This is of considerable interest, if it is realized that already at normal gravity a change from supine to upright position causes a significant reduction of cardiac output not only at rest but also during leg exercise (Bevegård, Holmgren and Jonsson 1960).

The improvement of circulatory tolerance to 3 G caused by exercise indicates a rise in the arterial pressure at head level. It is concluded that the shunting of arterial flow to the dependent portions of the body produced by the combined action of large hydrostatic pressures and strongly increased blood flow through working leg muscles did not occur at the expense of a sufficient arterial blood flow at head level. This indicates a substantial increase in the cardiac output.

References

ÅSTRAND, I. Aerobic work capacity in men and women with special reference to age. *Acta physiol. scand.* 1960 *49* Suppl. 169 1—92.

BARKER, S. B. and W. H. SUMMERSON, The colorimetric determination of lactic acid in biological materials. *J. biol. Chem.* 1941 *138* 535—554.

BEVEGÅRD, S., A. HOLMGREN and B. JONSSON. The effect of body position on the circulation at rest and during exercise with special reference to the influence on the stroke volume. *Acta physiol. scand.* 1960 *49* 279—298.

BJURSTEDT, H., Aeromedical Research in Sweden. *Medd. Flyg- Navalmed. Nämn.* Stockholm Congr. N 1957 1—4.

BJURSTEDT, H., L. E. HAMMON and G. STRÖM, Electrocardiographic heart-rate and subjective responses to prolonged gravitational stress and their relation to some dimensional and functional parameters of the circulatory system. *Acta physiol. scand.* 1959 *47* 97—108.

BROWN, J., G. E., E. H. WOOD and E. H. LAMBERT. Effects of tetra-ethyl-ammonium chloride on the cardiovascular reactions in man to changes in posture and exposure to centrifugal force. *J. appl. Physiol.* 1949 *2* 117—132.

GAUER, O. H. and H. L. THRON. Postural changes in the circulation. In *Handbook of Physiology Circulation.* Washington, D. C. Amer. Physiol. Soc. 1965 vol. III 409—2439.

GAUER, O. H. and G. D. ZUIDEMA, The physiology of positive acceleration. In GAUER O. H. and G. D. ZUIDEMA, *Gravitational Stress in Aerospace Medicine.* Boston Little Brown & Co 1961 115—133.

GRANATH, K. E. Some supplementary leads in clinical electrocardiography. *Acta med. scand.* 1948 Suppl. 209 pp. 463.

GÖTESTAM, J. and E. HELLMAN. A "human centrifuge" for research into physiological flight stresses. *Medd. Flyg- Navalmed. Nämn.* Stockholm 1955 *4* 3—13.

HENRY, J. P., O. H. GAUER, S. S. KETY and K. KRAMER, Factors maintaining cerebral circulation during gravitational stress. *J. clin. Invest.* 1951 *30* 292—300.

HOLMGREN, A. and K. H. MATTSSON. A new ergometer with constant work load at varying pedalling rate. *Scand. J. clin. Lab. Invest.* 1954 *6* 137—140.

HOLMGREN, A. and T. STRANDELL, On the use of chest-head leads for recording of electrocardiogram during exercise. *Acta med. scand.* 1961 *169* 57—62.

LAMBERT, E. H. The physiologic basis of "blackout" as it occurs in aviators. *Fed. Proc.* 1945 *4* p. 43.

RUSHMER, R. F. *Cardiovascular dynamics.* Philadelphia and London W. B. Saunders Company 1961 pp. 503.

SIEKER, H. O. Effect of acceleration on the heart. In GAUER O. H. and G. D. Zuidema, *Gravitational Stress in Aerospace Medicine.* Boston Little Brown & Co. 1961 5—60.

SJÖSTRAND, T. Changes in the respiratory organs of workers at an ore smelting work. *Acta med. scand.* 1947 *1*?? 687—69.

STRANDELL, T. Electrocardiographic findings at rest, during and after exercise in healthy old men compared with young men. *Acta. med. scand.* 1963. *174*. 479—499.

STRANDELL, T. Heart rate, arterial lactate concentration and oxygen uptake during exercise in old men compared with young men. *Acta physiol. scand.* 1964. *60*. 197—216.

STRÖM, G. The influence of anoxia on lactate utilization in man after prolonged muscular work. *Acta physiol. scand.* 1949. *17*. 440—451.

STURM, R. E. and E. H. WOOD, An instantaneous recording cardiotachometer. *Rev. Sci. Instr.* 1947. *18*. 771—776.

WAHLUND, H., Determination of the physical working capacity. *Acta med. scand.* 1948. Suppl. 215. pp. 78.

WOOD, E. H., E. H. LAMBERT, E. J. BALDES and C. F. CODE, Effect of acceleration in relation to aviation. *Fed. Proc.* 1946. *5*. 327—344.

WOOD, E. H., W. F. SUTTERER, H. W. MARSHALL, E. F. LINDBERG and R. N. HEADLEY, *Effect of headward and forward accelerations on the cardiovascular system.* WADD Techn. Rep. 60—634. Wright Patterson Air Force Base, Ohio. Jan. 1961. pp. 48.

Acta Physiol. scand. 1968. 72. 81—84

From the Department of Histology University of Umeå, Sweden

Glucose Tolerance in the Period Preceding the Appearance of the Manifest Obese-Hyperglycemic Syndrome in Mice

By

Ake Danielsson, Bo Hellman and Inge Bert Täljedal

Received 10 May 1967

Abstract

Danielsson A., B. Hellman and I. B. Täljedal. *Glucose tolerance in the period preceding the appearance of the manifest obese hyperglycemic syndrome in mice.* Acta physiol. scand. 1968. 72. 81—84

After intraperitoneal glucose injections in suckling mice there was higher frequency of glucosuria in those animals which later developed the obese-hyperglycemic syndrome. The experimental design had a particular advantage in screening for the early identification of mice which are homozygous for the obese-hyperglycemic gene. The observations also suggested that impairment of glucose metabolism represents a primary lesion in the obese-hyperglycemic syndrome. The hypothesis that the accumulation of fat later in life only reflects an adaptation of the adipose tissue to the high circulating levels of insulin in the obese-hyperglycemic mice deserves further attention.

The observation that human adiposity is often associated with an impaired glucose metabolism has focused attention on laboratory animals that show spontaneous development of obesity and hyperglycemia. The mice with the American variety of the obese-hyperglycemic syndrome have been most extensively investigated and found to be homozygous for a single recessive gene (Ingalls *et al.* 1950). For a proper understanding of the metabolic abnormalities in these animals, it is important to know whether the impaired glucose metabolism is secondary to the manifest obesity or a preceding phenomenon. In the present paper this problem has been studied by performing systematic glucose tolerance tests on suckling mice of different ages. The results were interpreted simply on the basis of the presence or absence of urinary glucose after an intraperitoneal glucose load. This means that the experimental technique may serve as a routine procedure for the early detection of the homozygotes which later develop the well-known characteristics of the obese-hyperglycemic syndrome.

Methods

Suckling mice of the strain with the recessive trait for th American variety of the obese-hyperglycemic syndrome (Roscoe B. Jackson Memorial Laboratories, Bar Harbor Maine USA) were used. All the experiments were performed between 10 and 11 a.m. in a room with utomatically ontrolled daylight 12 hrs from 8 m. In a preliminary study the possibility of inducing glucosuria by a single intraperitoneal dose of glucose was tested on about 400 animals representing different ages of 12 days or more. It wa checked that all animals displayed negative reaction to urinary glucose when tested with glucose oxidase strips (Clinistix® Ames Co. Inc., England) before the experiments. After the animals had been taken from their mothers nd weighed they were intraperitoneally injected with varying volumes of 10 % (w/w) glucose in distilled water. One hour later the presence of urinary glucose was checked with the glucose oxidase strips. After some practice it was easy to empty the urinary bladder both at the beginning of the experiment and 1 hou after the injection, simply by the application of a gentle pressure over the lower part of the abdomen.

In the main experiment 256 mice representing the following three agegroups were analysed 18±1 23±1 and 29±1 days. The amount of the intraperitoneal glucose load was calculated in terms of the body weight and hosen on the basis f the preliminary experiments, so as to be compatible with a frequency of about 50 per cent positive urinary glucose tests in each group. The experiments were performed in similar way t that described abov and the proced re was repeated after 24 hrs. Animals, which remained negative after these consecutive tests, were denoted — while positive tests were referred to as + or ++ depending upon whether they represented single or repeated observation. By inspecting and weighing all the mice one month lat they could easily be classified as being either homozygous (AO-mice) or non-homozygous (AN-mice) for the obese-hyperglycemic gene.

Results

There were no sex differences in the frequency of glucosuria after intraperitoneal administration of various doses of glucose. With greater ages of the suckling mice, the doses of glucose had to be gradually increased in relation to the body weight to establish glucosuria. While the intraperitoneal glucose load, which gave a frequency of about 50 per cent positive tests, corresponded to about 200 mg per 100 g body weight in 18-day-old animals, the corresponding figures for the 23- and 29-day-old mice were 250 and 300 mg

Table 1 The results of the intraperitoneal glucose tolerance tests on suckling obese-hyperglycemic mice (AO-mice) and their lean litter-mates (AN-mice) The urine was tested for glucose 1 hour after the injection on two consecutive days. Animals, which remained negative in these tests, have been denoted as — while positive urinary-glucose tests have been referred to as + or ++ depending upon whether they represent single or repeated observations. While the 18-day-old mice were injected with 200 mg per 100 g body weight, the 23-day-old mice received 250 mg and the 29-day-old mice 300 mg per 100 g body weight. The body weights for the AO- and AN-mice in each age group have been expressed as the mean values ± S.E.M.

Age in days	AN-mice				AO-mice			
	Body weight (g)	Tests for glucosuria			Body weight (g)	Tests for glucosuria		
		++	+	—		++	+	—
18	6.7±0.1	9	15	17	6.9 0.2	3	3	5
23	9.3±0.2	7	19	25	9.5±0.4	11	8	1
29	13.4±0.4	0	10	23	15.3±0.6	18	1	1

The presence of glucosuria after intraperitoneal glucose injections in suckling mice later classified as either homozygous or non-homozygous for the obese-hyperglycemic gene, can be seen in Table I. At an age of 18 days no significant differences were recorded in the frequency of positive urinary glucose tests between the AN- and the AO-mice. Five days later however there was a considerably higher frequency of glucosuria among the AO-mice (95 per cent, as compared to 51 per cent for the AN-mice $P < 0.01$) despite a normal body weight. When the mice were 29 days old, repeated positive tests for glucose were obtained in as many as 90 per cent of the AO-mice but in none of the AN mice after intraperitoneal injections of 300 mg glucose per 100 g body weight. For neither the AN-mice nor the AO-mice could any relationship be demonstrated between the body weight and the frequency of positive urinary glucose tests. At 23 days the 11 AO-mice with repeated positive tests had, for example, a body weight of 9.1 ± 0.6 g as compared with 9.8 ± 0.6 g for the 8 AO-mice found to have only single positive tests. At the same age the body weights of the 26 AN-mice displaying positive tests for glucosuria was 9.5 ± 0.4 g, as compared with 9.2 ± 0.3 g for the 25 AN mice with completely negative tests.

Discussion

The AO-mice are indistinguishable in external appearance from their lean litter mates during the first 4—6 postnatal weeks. The subsequent period is characterized by a rapid expansion of the subcutaneous depot fat, giving body weights double those of the controls (Täljedal and Hellman 1963, Hellman 1965). On the other hand, the hyperglycemia is known to develop progressively usually after the 12th and before the 18th week of life. The precise time of onset of hyperglycemia has been regarded as being conditioned by the presence or absence of genes other than "obese" in the genetic composition of the AO-mice (Mayer and Silides 1953). The present demonstration that an intraperitoneal glucose injection induces a significantly higher frequency of positive urinary glucose tests in the AO-mice as early as between the 3rd and 4th weeks of postnatal life suggests that impairment of the glucose metabolism represents a primary lesion in the obese-hyperglycemic syndrome. This interpretation is in accordance with the recent report of Stauffacher *et al.* (1967) that the AO-mice display an almost total absence of muscular response to intraperitoneally injected insulin. The multiplication of the pancreatic β cells in these animals (Gepts *et al.* 1960, Hellman 1965) with the resulting increase of circulating insulin-like activity (Christophe 1961) may explain why hyperglycemia will not be manifest until 2 months later and during the obese phase. The possibility that the accumulation of fat in the obese-hyperglycemic syndrome only reflects the adaptation of the adipose tissue to high local concentrations of insulin is an attractive hypothesis, which is in direct line with present views of the peripheral effects of this hormone. It has been suggested that a similar mechanism operates in the development of the obesity associated with mice bearing ACTH-secreting tumours (Hausberger and Ramsay 1959).

In view of the simplicity inherent in using commercially available glucose oxidase strips to demonstrate urinary glucose, we preferred to evaluate the glucose tolerance in a great number of animals only with respect to the presence or absence of glucosuria after intraperitoneal glucose injections. The possibility that differences in the absorption from the peritoneal cavity and the renal threshold for glucose may have contributed to the higher frequency of positive urinary glucose tests in the AO-mice makes it necessary to evaluate the early appearance of an impaired glucose metabolism also with conventional intravenous glucose tolerance tests. It should, however be emphasized that the present screening procedure is of particular advantage for the early identification of mice that will later develop the obese-hyperglycemic syndrome.

Supported by the Swedish Medical Research Council (12x-562-03)

References

CHRISTOPHE J. *Contribution à la biochimie des obésités expérimentales.* Editions Arch. Bruxelles 1961

GEPTS, W J CHRISTOPHE and J MAYER, Pancreatic islets in mice with the obese-hyperglycemic syndrome. Lack of effect of carbutamide. *Diabet* 1960 *9* 63—69

HAUSBERGER, F X. and A. J RAMSAY Islet hypertrophy in obesity of mice bearing ACTH-secreting tumours. *Endocrinology* 1959 *65* 165—171

HELLMAN, B., Studies in obese-hyperglycemic mice. *Ann. N Y Acad Sci.* 1965. *131* 541—558

INGALLS, A. M., M. M. DICKIE and G D SNELL, Obese, new mutation in the mouse *J Hered* 1950 *41* 317—318.

MAYER, J and D N SILIDES, A quantitative method of determination of the diabetogenic activity of growth hormone preparations. *Endocrinology* 1953 *52* 54—56.

STAUFFACHER, W A. E. LAMBERT D VECCHIO and A. E. RENOLD, Measurements of insulin activities in pancreas and serum of mice with spontaneous ("obese and "New Zealand obese) and induced (goldthioglucose) obesity and hyperglycemia, with considerations on the pathogenesis of the spontaneous syndrome. *Diabetologia* 1967 *3* 230—237

TÄLJEDAL, I B. and B. HELLMAN Morphological characteristics of the epididymal adipose tissue in different types of hereditary obese mice. *Path. et Microbiol.* (Basel) 1963. *26*. 149—157

Acta physiol. scand. 1968. 72. 85—99

From the Coagulation Research Laboratory and the Surgical Research Laboratory
Thoracic Clinic, Karolinska Institutet, Stockholm, Sweden

Effects of Thrombin Infusions upon ^{131}I labelled Fibrinogen in Dogs

By

SAM NORDSTRÖM and ERIC ZETTERQVIST

Received 11 May 1967

Abstract

NORDSTRÖM, S. and E. ZETTERQVIST *Effects of thrombin infusions upon ^{131}I-labelled fibrinogen in dogs* Acta physiol. scand. 1968. 72 85—99

The elimination of ^{131}I-labelled fibrinogen was followed in 5 groups of dogs, 1 after thrombin infusion, 2. after thrombin infusion in heparinized dogs, 3 after simultaneous infusions of epsilon aminocaproic acid (EACA) and thrombin, 4 after simultaneous infusions of Trasylol and thrombin and 5 controls. Platelet number fibrinogen concentration and radioactivity in plasma and fibrinogen were determined. Thrombin caused an immediate fall of both plasma and fibrinogen radioactivity The plasma radioactivity however soon started to increase. This secondary increase of plasma radioactivity seemed to be due to fibrinogen-fibrin degradation products. This indicates that the thrombin-induced intravascular coagulation gives rise to secondary fibrinolytic process. In heparinized dogs the coagulation-promoting effect of thrombin was inhibited. EACA and Trasylol in large doses, given simultaneously with thrombin, had no effect on the coagulation process but prevented the secondary plasma radioactivity increase. The fibrinogen-fibrin degradation products at different times were calculated. It was also possible to estimate the amount of synthetized fibrinogen at the different times. The highest mortality was obtained in the Trasylol-thrombin group. A probable explanation is that Trasylol is more effective than EACA in preventing the compensatory clot-dissolving fibrinolysis.

The effects of coagulation promoting substances such as thrombin and thromboplastin upon blood coagulation in vivo have been studied in many investigations (Warner *et al.* 1939 Quick *et al* 1959 Monkhouse and Milojevic 1960, Lewis and Szeto 1962 Kowalski *et al.* 1965) Thrombin and thromboplastin in appropriate amounts induce a syndrome characterized by fall in the concentration of plasma fibrinogen, in the number of circulating platelets and in the activity of factors V VIII prothrombin and serum factors (VII IX, X) These changes are reflected in prolongation of both the coagulation time and the prothrombin time. After i.v administration of thrombin and thromboplastin in dogs the appearance of an anticoagulant has also been demonstrated (Lewis and Szeto 1962 Kowalski *et al* 1965)

The present investigation deals with the elimination of ^{131}I-labelled fibrinogen after infusion of thrombin and the effects of simultaneously administered anticoagulant and antifibrinolytic substances.

Preliminary reports on this work have appeared (Nordström and Zetterqvist 1966, Nordström *et al* 1966)

Material

Fibrinogen

Dog fibrinogen was prepared from pooled mongrel dog citrated plasma according to Blombäck and Blombäck (1956)

^{131}I-labelled dog fibrinogen was prepared essentially according to McFarlane as described for human ^{131}I-fibrinogen (Blombäck *et al* 1966) The coagulability of the preparation before and after labelling was on the average 93 (range 88—98) and 90 (range 77—98) per cent, respectively The radioactivity in fibrin in per cent of that in fibrinogen was as mean 85 (range 71—95) per cent. The labelling was on the average 1.2 (range 0.3—2.6) atoms of iodine per mole of fibrinogen.

For a control experiment one batch of fibrinogen was also iodinated without the addition of isotope.

The iodinated fibrinogen was stored at —35 C for 2 days—2 weeks before it was injected.

Thrombin was the bovine preparation of Parke Davis and Co (Thrombin Topical®) containing 1,000 NIH units ml saline

Heparin (5,000 I.U./ml was the commercial product of Vitrum, Stockholm.

Epsilon aminocaproic acid (EACA 0 1 g/ml) was the commercial product Epsikapron® of KABI Stockholm.

Trasylol® kallikrein inhibitor prepared from bovine lung, containing 20 000 K. I. U./ml, was supplied by Bayer Farma AB, Stockholm.

Streptokinase (50,000 Christensen units per ml) was the commercial product Kabikinase® of KABI Stockholm.

Methods

Hematocrit was determined after centrifugation of heparinized capillary tubes for 8 min at 8,000 rpm. (Cellokrit, AB Lars Ljungberg & Co Stockholm)

Platelet counts were made with the method of Kristenson with the exception that blood was collected in tubes containing 5 mg EDTA disodium salt ethylendiamino-tetraacetic acid)

Fibrinogen concentration was measured essentially according to Blombäck and Blombäck (1956) as described by Bergström, Blombäck and Kleen 1960 One ml of citrated plasma was mixed with 0 5 ml of lysine ethylester hydrochloride solution (48 mg ml, adjusted to pH 6.5—6.5) To 2 ml of 0 11 M phosphate buffer (0.10 M KH_2PO_4 0.01 M Na_2HPO_4 pH 5 9 0 15 ml of thrombin solution 100 NIH units/ml) was added The clot was left standing for 2 hrs at room temperature, wound on silk cloth, washed, collected on a glass rod and dissolved in 5 ml of alkaline urea (40 % urea in 0.2 N NaOH After standing for hrs room temperature 0 5 ml of 0 3 M NaCl was added and the extinction read in Beckman DU photometer The extinction coefficient ($E^{1}_{1\,cm}$ = 16.5) at 282 mμ) for bovine fibrinogen was used Blombäck 1 58 I 5 does the amount of fibrinogen was measured with increasing concentrations of thrombin up to 20 NIH units per ml of the assay solution.

Fibrinolytic activity was determined as described by Bergström, Blombäck and Kleen (1960) In this method the difference between the fibrin concentration in samples with and without lysine ethylester hydrochloride gives the amount of lysed fibrin.

Radioactivity in plasma and in the coagulated fibrinogen was determined in well scintillation counter (EKCO N 610 A, England) with measurement error of less than 2 per cent at 3,000 counts. The samples were measured in polypropylene cups (height 55 mm, inner diam. 13 mm) For determination in plasma 1 ml of plasma was added to 3 ml of saline. For determination in fibrinogen, the radioactivity in 4 ml of the solution used for reading the fibrinogen concentration, was measured.

The radioactivity values for plasma and fibrinogen were plotted on logarithmic scale against time. From the 3rd day onwards an apparently straight line was obtained in the semi-logarithmic plot, indicating equilibration between the intra- and extravascular compartments and that apparent first order kinetics applied. The half-life time was calculated from the slope of the straight line connecting the points from the 3rd to the 5th or 6th day

Animal experiments

29 mongrel dogs 9—21 kgs of weight were injected i. with ^{131}I-labelled fibrinogen in doses giving an initial adioactivity of 40—60 μC × 10^{-4} per ml plasma. The dogs were given potassium iodide, ne tablet of 0.5 g daily, from 2 to 3 days before the injection. On the 5th or 6th day after jection of the labelled fibrinogen, thrombin infusion was given. The dogs were anesthetized by intravenous injection of 6 % penthobarbital sodium solution in doses around 25 mg pe kg b.w Varying doses of thrombin, diluted in 100 ml sterile saline, were infused during one hr through polyethylene catheter to central ein or to the right atrium.

The dogs were treated as follows

Group I 5 dogs were given thrombin infusions in doses of 50 to 250 NIH units per kg b.w respectively

Group II 2 heparinized dogs (1,000 units per kg b.w of heparin Vitrum) were given 150 units of thrombin per kg b.w

Group III 5 dogs were given 150 units of thrombin per kg and epsilon aminocaproic acid (EACA) in doses of 0.2 to 0.8 g per kg b.w., 1/3 of the dose as single injections before and after the thrombin infusion respectively and 1/3 diluted in saline as drip infusion simultaneously with the thrombin.

Group IV 8 dogs were given 100 to 150 units of thrombin per kg and Trasylol in doses of 6,500 to 100,000 units per kg b.w The Trasylol dose was administered in the same way as the EACA in group III.

Group V 9 dogs were used as controls I 4 of these the decay of the radioactivity in plasma and fibrinogen was followed up to 10 days for determination of the basic half-lif time. As controls regarding the influence of anesthesia and indw lling catheter 2 dogs were used. On the 6th day after injection of ^{131}I fibrinogen one of these dogs was only anesthetized, in the other also catheter was inserted, through which saline infusion was given during one hr Three dogs were given iodinated fibrinogen without isotope and followed during 10 days.

Blood samples were collected before and 15 min after the ^{131}I-fibrinogen injection, then on days 3 4 5 6 and occasionally 7 On the day of the experiment samples were taken before anesthesia, then at 15 30 60 min and at 2, 4 and 6 hrs after the start of the thrombin infusion.

The blood samples were drawn from leg ein into 3.8 % trisodium citrate (9 parts of blood to 1 part of citrate) in siliconized glass tubes for determination of fibrinogen concentration and radioactivity counting. For platelet counting the blood was collected in tubes containing 5 mg of EDTA and for hematocrit determination in heparinized capillary tubes.

Results

Already in the first samples taken 15 min after injection of ^{131}I fibrinogen there was difference of around 20 per cent between the total radioactivity of plasma and that of fibrinogen, determined as protein coagulable with thrombin (see figures) This difference is probably explained by the low specific activity of fibrin as compared with that of fibrinogen in the labelled preparation.

The results from the experiments re illustrated in Fig 1—7 and in Table I—III

The values are not corrected with respect to changes in hematocrit and to loss of blood by sampling

The total amount of blood collected by sampling during 9—10 days was around 200 ml in all dogs Of this volume 90 ml were removed on the day of the experiment. Because of different weights of the dogs the amount of radioactivity removed with each sample was calculated to between 0.5 and 2 per cent of the plasma radioactivity Correction for these losses of blood and radioactivity is not possible to make as the changes of blood and plasma volumes and other fluid losses are not known. Neither were corrections made for changes in the equilibration of the radioactivity after each sample. These changes must, however be small

Changes in plasma volume can not without objection be evaluated from hematocrit changes. During blood coagulation varying amounts of blood corpuscles may be trapped in clots and thus removed from the circulating blood.

During the first days after injection of labelled fibrinogen there was a tendency to a moderate and transient decrease in plasma fibrinogen concentration. In most dogs the initial fibrinogen level was again reached on the day of the thrombin injection. The platelet number showed inconsistent variations.

The fibrinolytic activity in 20 normal dogs was found to be 89 7 µg/ml/hr and the s.d. 19 6.

There were no variations in the fibrinogen concentration when the samples were tested with the standard concentration of thrombin or with larger amounts up to 20 NIH units in the assay solution.

Group I Thrombin infusion

Fig 1 shows the radioactivity curves obtained in one of the dogs given thrombin, 40 units per kg b.w In Table I the values for fibrinogen, degradation products and fibrinolytic activity are given for all dogs in this group.

After thrombin infusion there was an increased rate of disappearance of radioactivity in plasma and fibrinogen In the sample taken 30 min after the beginning of the infusion, the curves for whole plasma and fibrinogen started to separate The radioactivity of whole plasma increased and reached a maximum 2 hrs after the start of the thrombin infusion From this point the half life time in plasma was somewhat shorter than before the experiment. The radioactivity of the thrombin coagulable fibrinogen, however continued to decrease The decrease in fibrinogen concentration parallelled the decrease in radioactivity The platelet number was also rapidly lowered. The day after the experiment the fibrinogen concentration was again at the initial level. The number of platelets had not yet reached the initial value

The changes in the decay curves, fibrinogen concentration and platelet number in all the dogs of this group were of the same type but varied quantitatively depending on the amount of the thrombin infused. In one dog (no. 921) however the amount of radioactivity injected was too small to permit a reliable evaluation of the radioactivity measurements of the experiment samples.

The fibrinolytic activity in plasma was followed in 2 of the dogs (no. 809 921)

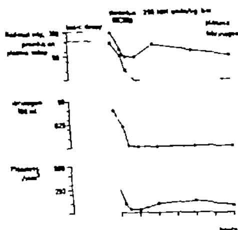


Fig. 1 Infusion of *large dose of thrombin* 5 days after i. injection of ^{131}I-fibrinogen. Dog no. 742. Plasma radioactivity before experiment 4.55 μC 10⁻ per mL.
Plasma and fibrinogen radioactivity fibrinogen concentration and platelet number

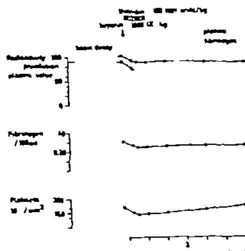


Fig 2. Infusion of a *medium dose of thrombi after k parturition.*
Dog no. 889 Plasma radioactivity before experiment 3.47 μC 10^{-8} per mL.

TABLE I. Thrombin infusion in dogs injected with ^{131}I-fibrinogen

	Dog nr	Control (before inj. of ^{131}I-fib.)	Thrombin NIH-units per kg b.w	Control (before experiment)	Time after start of thrombin infusion hours 1/4	1/2	1	2	4	6	day 1
Fibrinogen g/100 ml	921	0 19	50	0.35	0.28	0.30	0.23	0.24	0.25	0.24	0.26
	809	0.46	100	0.37	0.27	0.25	0.10	0 08	0.11	0 13	0.25
	787	0.75	150	0 72	0.68	0.61	0.06	†			
	103	0.67	150	0.63	0.62	0 51	0.22	0.20	0 24	—	0.50
	742	0.24	250	0.38	0.21	0.01	0	0	0	0	0 57
Calculated degraded fibrinogen-fibrin g/100 ml	809		100	0	0.03	0.06	0 04	0.15	0 10	0.11	
	787		150	0	0.05	0.11	0.10	†			
	103		150	0	0	0	0 04	0.28	0.27		
	742		250	0	0	0.03	0 14	0.26	0 20	0 13	
Fibrinolytic act ug/ml/hr pH 6 3	921		50	117	207	24	140	87	94		
	809		100	17	0	24	0	10	17		

and was found to be normal in one of them (no. 809) In the other dog there was slight increase immediately and one hr after the start of the thrombin infusion. Compared to the initial value the fibrinolytic activity was, however not much elevated.

Knowing the fibrinogen concentration and the radioactivity in plasma and fibrinogen at different times one can estimate the amount of degraded fibrinogen corresponding to the secondary increase in plasma radioactivity[1] In Table I the amounts of degradation products (calculated from the secondary plasma radioactivity increase) at each sampling time during the experiments are given. At the time of the "peak" this amount is in three dogs (no. 809 103 742) 52, 58 and 69 per cent respective y of the fibrinogen decrease up to that point. It seems that with increasing thrombin concentrations more of the lost fibrinogen will be regained as degradation products. The true amounts of degradation products are probably larger than the calculated ones, as part of them may escape rapidly from the circulation by excretion and other clearing mechanisms. On some occasions and mostly during the thrombin infusion negative values are obtained. This may be due to either incorrect measurements of the radioactivity or to screening out of labelled products from the plasma. This will cause a decrease in the difference between plasma and fibrinogen radioactivity

The results of the determination of fibrinogen concentration and radioactivity suggest that an increase in fibrinogen synthesis began soon after the start of the thrombin infusion. At four hrs after that point the synthesis will account for up to 50 per cent of the circulating fibrinogen.

One of the five dogs in this group (no. 787) died from occluding thrombo-emboli during the experiment

Group II Thrombin infusion in heparinized dogs

In Fig 2 the results in one of the 2 dogs, which were heparinized before the thrombin infusion, are shown. Heparin prevented most of the changes induced by thrombin in the previous group of dogs. A slight decrease in the platelet number was observed early after the start of the thrombin infusion. The other heparinized dog showed high fibrinogen values and very low platelet counts on the experiment day but the radioactivity curves were quite similar to those of the first heparinized dog

The fibrinolytic activity was not estimated.

As expected, the calculated amounts of degradation products were small.

Group III Thrombin infusion with epsilon-aminocaproic acid (EACA)

The results are shown in Fig 3—4 and Table II The doses of EACA were between 0 2 and 0 8 g per kg b w In one dog (no. 923) the initial radioactivity was too small to permit any study In the other four dogs the radioactivity decay curves

[1] For estimation of the amount of degraded fibrinogen the following formula was used

$$D_x = \frac{F}{f_o}\left[pl_x - pl_o - f_o \quad f \quad \right]$$

D_x = calculated degraded fibrinogen time

F = fibrinogen concentration at zero time

f_o, f_x = radioactivity fibrinogen

pl_o, pl_x = plasma radioactivity

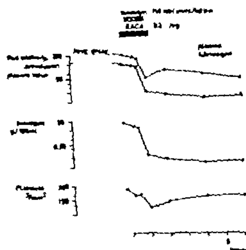


Fig. 3 Simultaneous infusions of small d se of EACA and medium d se f thrombin. Dog no. 788. Plasma radioactivity before experiment 10.7 μC 10⁻³ per ml.

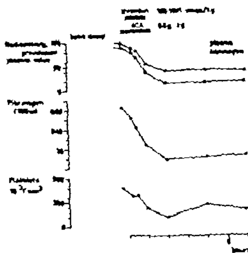


Fig. 4 Simultaneous infusions of large d se f EACA and medium dose f thrombin. Dog no. 244 Plasma radioactivity before experiment 4 14 μC 10⁻³ per ml.

initially showed the same fall as that obtained with thrombin alone. Three of the four dogs got a thrombin dose of 150 units per kg b.w and 0.2, 0 4 and 0.8 g per kg b.w of EACA, respecti ely In the dogs given 0.2 and 0 4 g per kg b.w there was a definite secondary increase in the plasma radioactivity following the initial drop In the dog which got the largest dose (0.8 g per kg b.w) the secondary plasma "peak was abolished (Fig. 4) One of the dogs in this group got a smaller thrombin dose (100 units per kg b w) than the others and 0.4 g per kg of EACA. The secondary plasma radioactivity increase was definitely smaller which might be related to the thrombin dose.

The fibrinogen concentration and platelet number changed in roughly the same w y as with thrombin alone The fibrinolytic activity was determined in three dogs nd found to be within normal limits.

At 2 hrs after the start of the thrombin infusion the calculated amounts of degradation products in dogs 788 204 and 244 were 31 51 and 22 per cent, re spectively of the lost fibrinogen. Although in the dog with the largest EACA dose the secondary plasma peak seemed to be inhibited, as much as 22 per cent of the fibrinogen loss was calculated as degradation products.

All dogs in this group survived.

G oup II Th mbin nfusi n with T asylol

Table III show the results obtained when Trasylol was gi en together with thrombin in 8 dogs in doses from 6,500 to 100 000 units per kg b w In one dog gi en 10 000 units per kg b w the curves beha ed as in the dogs given thrombin alone I two dogs given 20,000 and 50 000 units per kg b.w., respectively the decay curves

Table II. Simultaneous infusions of EACA and thrombin in dogs injected with ^{131}I-fibrinogen

	Dog nr	Control (before inj. of ^{131}I fib.)	EACA g/kg b.w	Thrombin NIH-units per kg b.w	Control (before experiment)
Fibrinogen g/100 ml	788	0.38	0.2	133	0.48
	808	0.42	0.2	100	0.38
	923	0.38	0.4	150	0.34
	204	0.[illegible]0	0.4	150	0.46
	244	0.71	0.8	150	0.62
Calculated degraded fibrinogen-fibrin g/100 ml	788		0.2	133	0
	808		0.2	100	0
	204		0.4	150	0
	244		0.8	150	0
Fibrinolytic ug/ml/hr pH 6.3	788		0.2	133	207
	808		0.2	100	77
	923		0.4	150	100

Table III. Simultaneous infusions of Trasylol and thrombin in dogs injected with ^{131}I-fibrinogen

	Dog nr	Control (before inj. of ^{131}I-fib.)	Trasylol K.I.E. per kg b.w	Thrombin NIH-units per kg b.w	Control (before experiment)
Fibrinogen g/100 ml	741	0.39	6,500	100	0.66
	885	0.44	10 000	150	0.44
	890	0.25	20 000	150	0.38
	911	0.31	20 000	150	0.28
	887	0.29	30,000	150	0.30
	203	0.53	[illegible]0,000	150	0.3[illegible]
	933	0.38	60 000	150	0.40
	243	0.34	100,000	150	0.47
Calculated degraded fibrinogen-fibrin g/100 ml	741		6,[illegible]00	100	0
	885		10 000	150	0
	890		20,000	150	0
	911		[illegible]0,000	150	0
	887		30,000	150	0
	203		[illegible]0 000	150	0
	243		100 000	150	0
Fibrinolytic act. ug ml/hr pH 6.3	885		10,000	150	0
	933		60 000	150	84
	243		100 000	150	7

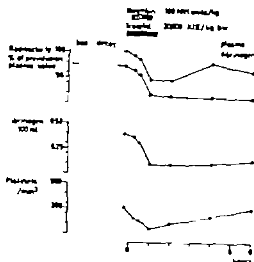
Values in ( ) are obtained after starting treatment with streptokinase

Time after start of thrombin infusion

hours						day
1/4	1/2	1	2	4	6	1
0.44	0.42	0.13	0.09	0.05	0.06	0.17
0.27	0.24	0.18	0.16	0.15	0.16	0.34
0.19	0.12	0.02	0	0	0.05	0.31
0.41	0.40	0.31	0.23	0.20	0.19	0.23
0.51	0.42	0.23	0.09	0.11	0.13	0.34
0	0.01	0.06	0.20	0.19	0.11	
0	0	0	0	0.03	0.05	
0.02	0.02	0.04	0.13	0.11	0.08	
0	0.03	0.06	0.12	0.12	0.10	
0	0	0	0	0		
0	4	30	17	4	0	
0	—	—	—	30		

Time after start of thrombin infusion

hours						day
1/4	1/2	1	2	4	6	1
0.53	0.40	0.33	†			
0.37	0.34	0.09	0.09	0.11	0.12	0.35
0.33	0.29	0.08	0.07	0.08	0.11	0.22
0.18	0.07	0	0	(0)	†	
0.25	0.21	0.12	0.08	(0.11)	(0.14)	(0.30)
0.33	0.28	*0.21*	*0.21*	0.19	*0.24*	*0.29*
0.35	0.28	0.16	0.14	0.15	0.15	0.28
0.51	0.41	0.26	0.22	0.26	0.25	0.55
0.02	0.01	0.05	†			
0.08	0.05	0.06	0.20	0.18	0.12	
0	0.02	0.01	0.02	0.20	0.12	
0	0	0	0.04	(0.10)	†	
0.05	0.04	0.05	0.13	(0.02)	0	
0	0	0	0.01	0.06	0	
—	0	0	0	0	0	
0	87	0	30	100	44	
117	83	114	104	60	200	
0	33	34				



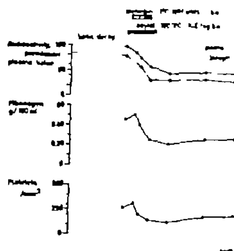


Fig. 5 Simultaneous infusions of *m dl m d se f Trasylol* and *a medium dose of thrombin*.
Dog no. 890. Plasma radioactivity before experiment 4.85 µC 10^{-3} per ml.

Fig. 6 Simultaneous infusions of a *large dose of Trasylol* and *medium dose of thrombin*.
Dog no. 243 Plasma radioactivity before experiment 6 67 µC 10^{-3} per ml.

for whole plasma and fibrinogen were seen to diverge from 2 hrs after the start of the thrombin infusion. At 4 hrs after the start there was a definite increase in the plasma radioactivity which with the smaller dose (20,000 units) was of the magnitude seen after thrombin alone (Fig 5) and with 50 000 units per kg b w was smaller However in comparison with the effects obtained in group I the secondary plasma peak here seemed to appear later Finally in the dog which got the largest dose (1000 000 units per kg b w) the decay curves of plasma and fibrinogen after he initial decrease followed each other (Fig 6) The changes in fibrinogen con u and platelets were of the same type as in the groups already mentioned In three dogs (no. 243 886 933) the fibrinolytic activity was measured in one dog (no. 933) a slight increase was noted immediately after the start of the thrombin infusion Six hrs later the fibrinolytic activity was somewhat more elevated. In the other dogs the activity wa not increased. Two hrs after the start of the thrombin infusion, the calculated amounts of degradation products in dogs 886 and 88 were 57 and 54 per cent, respectively of the fibrinogen loss. In four other dogs less than 13 per cent were found at that time Two hrs later the corresponding values in dogs 886, 890, 205 and 243 were 51 58, 34 and 0 per cent respectively Because of error in the radioactivity counting it was not possible to calculate the degradation products in dog 933

Two dogs (no 911 and 887) given medi m doses of Trasylol got severe symptoms of pulmonary thrombo-emboli at about 3 hrs after the start of the thrombin infusion. Both dogs were given streptokinase (250,000 Christensen units in 100 ml of saline) as intravenous infusion during 20 min. One of these dogs soon got pulmonary oedema and expired. At autopsy no emboli were found. The other one

recovered quickly after the start of the streptokinase infusion. At autopsy one week after the experiment no emboli were found in the pulmonary vessels or the heart. Other effects of streptokinase in these two dogs will not be commented on here.

Another dog (no 741) died during the experiment. The dog which got the largest Trasylol dose (100 000 K.I.U. per kg b.w.) died 24 hrs after the experiment. Autopsy in both these dogs showed occluding, firm thrombo-emboli in the right heart and the pulmonary artery.

Group I. Controls

In 5 dogs the radioactivity decay in plasma and fibrinogen was followed up to 10 days. It was found that the half-life time could be estimated from the values obtained from the 3rd day after the fibrinogen injection and onwards. The mean half life times for [131] I-labelled fibrinogen in 26 of the experiment dogs as measured in plasma and in coagulated fibrinogen were 2.0 and 1.9 days (ranges 1.2—3.0 and 1.2—2.8) respectively. The values for fibrinogen are in good agreement with those obtained for I-fibrinogen in dogs by other authors (Adelson *et al.* 1961, Lewis, Ferguson and Schoenfeld 1961).

In two of the three dogs given I-fibrinogen without isotope, there was a transient fall in the platelet number and a simultaneous rise in the fibrinogen concentration.

Fig. 7 shows the results in one of the control dogs, which on the experiment day was only anesthetized and given a saline infusion instead of thrombin. In another dog besides anesthesia and saline infusion also a catheter was inserted. The half life time for [131]I-fibrinogen was not significantly influenced by anesthesia and saline infusion or insertion of a catheter. Immediately after induction of anesthesia there was in both dogs a slight to moderate decrease in the platelet number and in one dog a short-lasting decrease in the fibrinogen level. The calculated fibrinogen degradation products in these dogs were small.

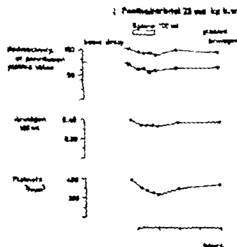


Fig. 7. Control. Effect of anesthesia and saline infusion.

Dog no. 105. Plasma radioactivity before experiment 3.83 μC. 10⁻ per ml.

Among the control dogs the fibrinolytic activity was evaluated only in one dog and was not found to be elevated.

The calculations of the fibrinogen degradation products were made without corrections for changes in plasma volume and basic radioactivity decay As mentioned before we had not estimated changes in the plasma volume. The basic radioactivity decay during the whole experiment cannot exceed 6 to 7 per cent

Discussion

In recent years disseminated intravascular coagulation has been discussed as cause of acute bleeding disorders (Hardaway and McKay 1963 McKay 1965 Verstraete *et al* 1965 Rodriguez Erdmann 1965) The coagulation changes found in patients where this syndrome has been confirmed by clinical or autopsy findings, agree well with those obtained in experimental conditions, where the coagulation system has been activated. As a decrease in the plasma fibrinogen level is one of the most striking changes after experimentally induced activation of the coagulation system, it should be of interest to follow the elimination of isotope labelled fibrinogen under such conditions.

In the fibrinogen preparations used for labelling the coagulability i.e. the percentage of protein coagulable with thrombin, was on the average 93 per cent. The determination of the coagulability was made with ultraviolet spectrophotometry of fibrinogen and fibrin in alkaline urea solution at 282 mμ By iodination the coagulability decreased a few per cent.

Assuming that the amino acid composition of dog fibrinogen is similar to that of human and bovine fibrinogen (Henschen and Blombäck 1964) one could calculate that the dog fibrinopeptides (Blombäck *et al.* 1966) contain about 1 per cent of the amino acid residues which absorb light in the ultraviolet at 282 mμ. One would consequently expect pure dog fibrinogen to have a coagulability of 99 per cent. In our batches it is then likely that on the average 6 per cent represent other proteins or denatured fibrinogen.

The specific radioactivity in fibrin was on the average 80 per cent of that in fibrinogen. The difference in coagulability of around 10 per cent as determined by ultraviolet spectrophotometry and by radioactivity measurement, respectively can be explained by an uneven iodination of the fibrinogen with concomitant loss of coagulability of the overiodinated molecules or by impurities in the preparation of other proteins, being preferentially labelled with the isotope. Thus, on a weight basis, the non-coagulable products might be labelled to a higher degree than the coagulable fibrinogen. These in vitro findings are well in accordance with the difference in vivo between the radioactivity in plasma and fibrinogen, which was noticed in the initial samples in all dogs.

Thrombin given as intravenous infusions in doses from 100 to 250 NIH units per kg b.w and hr caused a rapid decrease in the radioactivity in plasma as well as in the fibrinogen fraction. One to 1 1/2 hrs after the end of the thrombin infusion

there was an increase in the plasma radioactivity to a more or less pronounced peak whereas the radioactivity in the fibrinogen fraction as well as the concentration of fibrinogen remained at low levels. Because of the remaining low radioactivity of fibrinogen as well as the low concentration of thrombin coagulable fibrinogen, the secondary plasma peak cannot be explained by labelled native fibrinogen. The plasma radioactivity increase could, however be explained by ^{131}I-labelled degradation products of fibrinogen. The question then arises, whether such products are formed directly from fibrinogen during the influence of thrombin or if they are products of a lytic digestion of fibrin, formed in the coagulation process.

Of the two peptides (fibrinopeptide A and B) split off from the fibrinogen molecule during coagulation, only the fibrinopeptide B has the capacity of binding iodine. When after labelling of fibrinogen, the fibrinopeptide B was isolated, its radioactivity content was estimated to about 5—7 per cent of the total label (Zetterqvist 1967). Most of the radioactivity then must be found in the rest of the fibrinogen molecule i.e. the part which forms the fibrin. As the plasma radioactivity decreases very rapidly on thrombin infusion, fibrinogen must be withdrawn from the circulation. We therefore believe that practically all of the increased plasma radioactivity reflects the appearance in the circulating blood of degradation products of fibrin, obtained by a fibrinolytic process, secondary to intravascular coagulation. Like Kowalski *et al.* (1965) we could not find any significantly increased fibrinolytic activity in the circulating blood. These authors, who also studied the influence of thrombin upon the turnover of I labelled fibrinogen in dogs, thought like Kowarzyk and Kotschy (1962) that plasminogen adsorbed on the fibrin clot was activated locally. They also noticed a decrease of antiplasmin activity after thrombin infusion.

The calculated amounts of fibrinogen degradation products seem to reflect roughly the intensity of the fibrinolytic process. As was earlier mentioned and is illustrated in the figures the decay curve for plasma, after the peak mentioned, showed a steeper slope than the basic curve. This effect can possibly be given by fibrinogen degradation products, having a shorter half life time than fibrinogen itself. The reappearance of labelled fibrinogen on the day after the experiments could be explained by fibrinogen, reentering the blood from the extravascular space.

When heparin was given prior to the thrombin infusion the coagulation promoting effect of thrombin was, as expected, completely abolished. Of the two drugs with antifibrinolytic effect, Trasylol and EACA, Trasylol has besides this effect also an anticoagulant activity. This latter effect, however is directed against the initial phase of coagulation and not toward thrombin (Amris 1964, Blombäck, Blombäck and Olsson 1964). Trasylol, therefore had no effect on the thrombin-induced disappearance of fibrinogen in the dogs.

With sufficient concentrations of Trasylol and EACA it seemed possible to delay or inhibit the secondary plasma radioactivity increase after thrombin. With increasing doses of both substances it was found possible to decrease the amount of degradation products. This effect of Trasylol and EACA gives further support to

the hypothesis that the secondary plasma radioactivity increase is caused by fibrin split products. This is also upheld by the results of preliminary immunoelectrophoretic studies (Berglund 1966). Even when the largest dose of EACA was given, we could, however, demonstrate about 20 per cent of the lost fibrinogen as degradation products. When the largest dose of Trasylol was given together with thrombin, no degradation products were demonstrable. This may partly be due to a higher antifibrinolytic activity on molar basis of Trasylol.

Theoretically the combination Trasylol-thrombin respectively EACA-thrombin ought to give a higher frequency of thrombotic complications than thrombin alone. The reason is that the clots formed by thrombin cannot be solved, as the compensatory fibrinolytic process is inhibited. Although our series are too small to permit definite conclusions, we got a higher mortality rate in the Trasylol-thrombin group than in the dogs given thrombin alone. In the EACA-thrombin group all dogs survived. One might speculate that this difference in mortality between Trasylol and EACA treated dogs could be due to the fact that EACA does not, as Trasylol, directly inhibit plasmin. The inhibition of fibrinolysis with EACA might be slower (Godal and Theodor 1965).

This investigation was supported by grants from the Swedish Medical Research Council (grant no. K 67 19 X 520-03), the Swedish National Association against Heart and Chest Disease and from Bayer Firma AB, Stockholm.

References

ADELSON, E., J. J. RHEINGOLD, O. PARKER, A. BUENAVENTURA and W. H. CROSBY: Platelet and fibrinogen survival in normal and abnormal states of coagulation. *Blood* 1961, *17*, 267—281.
...sen, C. J.: Inhibition of fibrinolytic and thromboplastic activity by Trasylol. *Abstr. Xth Congr. Int. Soc. Haemat.* 1964, G 72. (*Scand. J. Haemat.* 1966, 3, 19—32.)
...und, G.: personal communication 1966.
...strröm, K., B. BLOMBÄCK and G. KLEEN: Studies on the plasma fibrinolytic activity in case of liver cirrhosis. *Acta med. scand.* 1960, *168*, 291—305.
BLOMBÄCK, B.: On the properties of fibrinogen and fibrin. *Arkiv Kemi* 1958, *12*, 99—113.
BLOMBÄCK, B. and M. BLOMBÄCK: Purification of human and bovine fibrinogen. *Arkiv Kemi* 1956, *10*, 415—443.
BLOMBÄCK, B., M. BLOMBÄCK, N. J. GRÖNDAHL and E. HOLMBERG: Structure of fibrinopeptides—its relation to enzyme specificity and phylogeny and classification of species. *Arkiv Kemi* 1966, *25*, 411—428.
BLOMBÄCK, B., M. BLOMBÄCK and P. OLSSON: Action of a proteolytic enzyme inhibitor on blood coagulation in vitro. Pers. comm. 1961. (*Neue Aspekte der Trasylol-Therapie*. F. K. Schattauer-Verlag, Stuttgart 1966, pp. 33—45.)
BLOMBÄCK, E., L. A. CARLSON, S. F...in and E. ZETTERQVIST: Turnover of 131I-labelled fibrinogen in man. *Acta med. scand.* 1966, *179*, 557—574.
GODAL, H. C. and I. THEODOR: In vitro inhibition of streptokinase induced proteolysis by Trasylol and by epsilon aminocaproic acid (EACA). *Scand. J. clin. Lab. Invest.* 1965, *17*, 199—208.
HARDAWAY, R. M. and D. G. McKAY: The syndromes of disseminated intravascular coagulation. *Rev. Surg.* 1963, *20*, 297—328.
HENSCHEN, A. and B. BLOMBÄCK: Amino acid composition of human and bovine fibrinogen and fibrin. *Arkiv Kemi* 1964, *22*, 347—354.
KOWALSKI, E., A. Z. BUDZYNSKI, M. KOPEC, Z. S. LATALLO, B. LIPINSKI and Z. WEGRZYNOWICZ: Circulating fibrinogen degradation products (FDP) in dog blood after intravenous thrombin infusion. *Thromb. Diath. haemorrh.* (Stuttg.) 1965, *13*, 12—24.

Kowarzyk, H. and M. Kotschy cited by Kowalski et al. 1965.

Lewis, J. H., E. E. Ferguson and C. Schoenfeld, Studies concerning the turnover of fibrinogen I[131] in the dog. *J. Lab. clin. Med.* 1961 *58* 247—258.

Lewis, J. H. and I. L. F. Szeto, Effects of intravenous tissue thromboplastin in dogs: development of an anticoagulant. *J. Lab. clin. Med.* 1962. *60* 261—273.

McKay D. G. *Disseminated intravascular coagulation.* Harper & Row Publ. New York. 1965.

Monkhouse, F. C. and S. Milojevic, Changes in fibrinogen level after infusion of thrombin and thromboplastin. *Amer. J. Physiol.* 1960. *199.* 1165—1168.

Nordström S., B. Blombäck, M. Blombäck, P. Olsson and E. Zetterqvist. Experimental investigations on the antithromboplastic and antifibrinolytic activity of Trasylol. *Ann. N. Y. Acad. Sci.* In press.

Nordström, S. and E. Zetterqvist. The elimination of [131]I-labelled fibrinogen after experimentally induced intravasal coagulation in dogs. *Lect. Swed. Med. Soc.* March 1966.

Quick, A. J., C. V. Hussey, J. Harris and K. Peters, Occult intravascular clotting by means of intravenous injection of thrombin. *Amer. J. Physiol.* 1959 *197* 791—794.

Rodriguez-Erdmann F. Bleeding due to increased intravascular blood coagulation. *N. Engl. J. Med.* 1965. *273* 1370—1378.

Warner, E. D., K. M. Brinkhous, W. H. Seegers and H. P. Smith, Further experience with the use of thrombin as a hemostatic agent. *Proc. Soc. exp. Biol.* (N.Y.) 1939 *41* 655—657.

Verstraete, M., C. Vermylen, J. Vermylen and J. Vandenbroucke, Excessive consumption of blood coagulation components as cause of hemorrhagic diathesis. *Amer. J. Med.* 1965 *38* 899—908.

Zetterqvist E. To be published.

Acta physiol. scand. 1968. 72 100—107

From the Department of Pharmacology University of Göteborg, Sweden

Studies on the Dual Action of Guanethidine in Sympathetic Nerves

By

PER LUNDBORG and ROBERT E. STITZEL

Received 12 May 1967

Abstract

P LUNDBORG and R. E. STITZEL. *Studies on the dual action of guanethidine in sympathetic nerves* Acta physiol. scand. 1968. 72 100—107

Evidence is presented indicating that guanethidine inhibits both the transport of amines through the nerve cell membrane and an uptake mechanism present in amine-storing granules. In vivo the onset of membrane pump blockade is the more rapid process, but inhibition of granular uptake is more long lasting. In vitro studies demonstrate that guanethidine can inhibit the uptake of ^{14}C-adrenaline into bovine adreno-medullary granules. The depletion of ^{3}H-metaraminol seen after guanethidine administration may result from a reserpine-like action on the amine storage granules and/or a protriptyline-like action on the membrane pump. It is suggested that the membrane pump inhibiting ability possessed by guanethidine, but not reserpine may account for the sympathomimetic activity and exogenous amine potentiation which often accompanies guanethidine but not reserpine administration.

Several authors (Sheppard and Zimmerman 1959 Cass, Kuntzman and Brodie 1960) have shown that guanethidine can deplete peripheral tissues, with the exception of the adrenal medulla (Athos *et al* 1962) of their catecholamine content. However there seems to be no correlation between the degree of sympathetic blockade and the extent to which amine stores are diminished. Guanethidine can produce a complete sympathetic blockade before amine levels are reduced (Cass and Spriggs 1961) and this blockade disappears before the amine content is noticeably repleted (Sanan and Vogt 1962)

It has been suggested that the neuronal blocking action of guanethidine may be due to an effect on a small functionally important pool of catecholamines in the amine-storing granules (Carlsson 1966) A blockade of amine uptake into this pool rather than a depletion of catecholamine stores might be a possible mechanism by which guanethidine causes sympathetic nerve blockade An action of guanethidine on adrenergic nerve granules has been proposed by Lindmar and Muscholl (1961) and was recently demonstrated *in vitro* by Shore and Giachetti (1966) However a detailed analysis of the subcellular sites of action of guanethidine has not been performed. The present work was undertaken to study *in vivo* as well as *in vitro* effects of guanethidine on the uptake function of amine-storing particles.

Methods

In vivo experiments Mice divided into groups of six, were used in all experiments. In one study mice were given guanethidine 50 mg/kg i.p. and at various intervals after drug administration the animals were injected with ^{3}H-α-methyl noradrenaline (^{3}H-α MeNA) 100 µg/kg i.v. (30 mc/mM) Control and experimental animals were killed 15 min after receiving the labelled amine.

Other groups were given ^{3}H-metaraminol, 40 µg/kg (100 mc/mM) i.v. Control groups received no further treatment and were killed either 30 min or 24 hrs late. The experimental groups were injected with either reserpine (0.5 mg/kg, i.v.) protriptyline (10 mg/kg, i.v.) or guanethidine (10 mg/kg, i.v.) 15 min or 24 hrs after the ^{3}H-metaraminol (^{3}H MA) injection. They were killed after another 15 min (short interval) or 45 min (24 hr interval). For further details, see Results section.

All animals were sacrificed by decapitation. The hearts were removed and homogenization performed in an ice bath using a plastic pestle. The homogenization medium was 0.25 M sucrose containing 0.005 M phosphate buffer pH 7.4 and 0.001 M $MgCl_2$. A coarse fraction was obtained by centrifugation at 4° C at 2000 × g for 10 min. The resulting supernatant was then centrifuged at 100,000 × g for 60 min in a Spinco model L Ultracentrifuge providing two more fractions particulate and high speed supernatant. After protein precipitation of the various fractions the samples were placed on an ion exchange column (Dowex 50 W X4). The column was washed with 40 ml of glass-distilled water and the ^{3}H MA or ^{3}H-α-MeNA was then eluted with 1 N HCl. Further details of the analytical procedure has been previously described (Bhrel and Lundborg 1967). All animals were kept at 30° C.

In vitro experiments. Amine granules from cow adrenal medullae were prepared essentially as described by Hillarp (1958). The granules were resuspended in 0.3 M sucrose and stored at 0° C for use on the same and the next day. An aliquot (50 µl) of the granule suspension, corresponding to about 125 µg of catecholamines, 20—25 µg of which are free amines released from the granules at their suspension, was transferred to 1.0 ml of an incubation mixture (at 0° C) containing glycyl-glycine (0.31 M) ATP (0.0025 M) and $MgCl_2$ (0.0025 M). C^{14}-adrenaline was added to the incubation medium. The final concentration of labelled plus unlabelled drug was about 3.0 × 10 M. This figure includes the 20—25 µg of amines released from the granular suspension. Varying concentrations of guanethidine were added to some of the incubation flasks. Incubations were performed without shaking at 0° or 31° C for 30 min.

Substances used (±) ^{3}H-MA and (±) ^{3}H-α-Me-NA were prepared by the research laboratory of Hässle Ltd in cooperation with this department (Hallhagen and Waldeck, to be published). C^{14}-adrenaline was obtained from New England Nuclear Corp. Reserpine and guanethidine were generously supplied by Swedish Ciba Ltd and protriptyline by Dr. C. A. Stone of the Merck Institute for Therapeutic Research.

Data were calculated for significance using an analysis of variance.

Results

Uptake of ^{3}H-α-MeNA by the different fractions of the mouse heart at various intervals after guanethidine injection. Guanethidine in a dose of 50 mg/kg was injected intraperitoneally into mice. At various intervals after guanethidine administration the animals were given 100 µg/kg of ^{3}H-α-MeNA i.v. and killed 15 min later. The total uptake of ^{3}H-α-MeNA as well as its subcellular distribution were studied.

Guanethidine caused a pronounced blockade of the uptake of ^{3}H-α-MeNA into the heart during the first 4 hrs (Fig. 1). At the 12 hr interval the uptake had partially recovered and 24 hrs after guanethidine administration the total uptake of ^{3}H-α-MeNA was almost the same as in control animals. Although there was a considerable decrease in total uptake of ^{3}H-α-MeNA 1/2 h after guanethidine administration, a calculation of the amount of ^{3}H-α MeNA in the particulate fraction

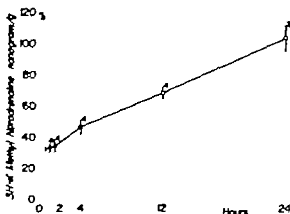


Fig. 1
Effect of guanethidine on the uptake of ^{3}H-α-methyl noradrenaline in the mouse heart. Animals were pretreated with guanethidine (50 mg/kg i.p.) and at various intervals they received ^{3}H-α-methyl noradrenaline (100 μg/kg i.v.). All animals were sacrificed 15 min after receiving the labelled compound. Vertical bars indicate standard error of the mean.

TABLE I Influence of guanethidine on the uptake and subcellular distribution of ^{3}H-α-methyl noradrenaline (^{3}H-α-MeNA) in the mouse heart

Treatment	Time after drug administration (hr)	^{3}H-α-MeNA-ng/g Particulate	Supernatant	$\frac{P}{P+S} \times 100$
None	0	25.25 ± 1.15	37.49 ± 2.17	40.4 ± 1.5
Guanethidine	1/2	6.60 ± 0.24	12.28 ± 0.19	35.0 ± 1.0
(50 mg/kg)	1	6.33 ± 0.78	13.25 ± 0.68	32.0 ± 2.0
	2	7.24 ± 0.88	13.25 ± 1.53	35.3 ± 1.3
	4	8.26 ± 0.69	17.47 ± 1.55	32.0 ± 0.49
	12	9.96 ± 0.64	30.34 ± 1.93	2[illegible].8 ± 1.7
	24	24.[illegible] ± 1.59	44.38 ± 2.97	35.7 ± 1.3

^{3}H-α-MeNA was given i.v. and the animals were sacrificed 15 min later. The ratio $\frac{P}{P+S} \times 100$ indicates the per cent of amine found in the particulate fraction relative to the combined particulate plus supernatant fractions. Each determination was performed on 6 pooled hearts and each value represents the mean (± S.E.M.) of at least 4 determinations.

as a percentage of that found in the particulate plus supernatant fractions show that there was only a moderate change in the subcellular distribution (Table I). Alteration in the distribution of ^{3}H-α-Me-NA was most pronounced at the 12 hr interval, but was still evident 24 hrs after the guanethidine administration ($P < 0.05$).

Comparison between the effect of guanethidine, reserpine, protriptyline and reserpine plus protriptyline on the release of ^{3}H-MA from subcellular fractions of the mouse heart

When given 15 min after ^{3}H-MA, reserpine had no depleting effect on either the particulate or on the soluble fractions (Table II). Protriptyline caused a significant ($P < 0.001$) decrease in the ^{3}H-MA content of the soluble fraction, but did not

TABLE II. Effect of reserpine, protriptyline and guanethidine on the subcellular distribution of ³H metaraminol (³H MA) in the mouse heart.
Animals were given reserpine, protriptyline or guanethidine i.p. either 15 min before (30 min interval) or 45 min before (24 hr interval) sacrifice. The values given are means ± S.E.M. Each experiment was performed on 6 pooled hearts

Time after ³H-MA	Treatment	H-MA—ng/g		$\frac{P}{P+S} \times 100$	Number of experiments
		Particulate	Supernatant		
	Control	7.82 ± 0.52	37.50 ± 0.96	17.2 ± 0.89	10
	Reserpine (10 mg/kg)	8.00 ± 0.44	36.64 ± 1.09	18.0 ± 1.08	6
30 min	Protriptyline (10 mg/kg)	7.70 ± 0.49	24.43 ± 1.62	24.0 ± 0.95	6
	Guanethidine (10 mg/kg)	4.71 ± 0.45	24.98 ± 2.44	15.9 ± 0.65	4
	Reserpine + Protriptyline	0.47 ± 0.06	4.28 ± 0.24	9.9 ± 0.75	4
	Control	7.12 ± 0.30	21.40 ± 0.88	25.0 ± 0.60	10
	Reserpine (10 mg/kg)	2.91 ± 0.31	15.78 ± 1.30	16.3 ± 1.88	7
24 hrs	Protriptyline (10 mg/kg)	6.13 ± 0.36	17.95 ± 1.32	25.9 ± 0.98	8
	Guanethidine (10 mg/kg)	2.50 ± 0.26	11.76 ± 0.65	17.5 ± 1.45	4
	Reserpine + Protriptyline	0.21 ± 0.02	1.49 ± 0.10	12.1 ± 0.99	4

affect the particulate fraction. Guanethidine produced a pronounced decrease in the amount of ³H MA in the particulate ($P < 0.001$) as well as in the supernatant ($P < 0.001$) fraction.

24 hrs after ³H MA administration reserpine caused a pronounced decrease ($P < 0.001$) in the amount of ³H MA in the particulate fraction and also a smaller but significant ($P < 0.001$) decrease in the supernatant fraction (Table II). Protriptyline had no effect on the ³H MA content of the particulate fraction, but produced a small decrease in the supernatant fraction ($P < 0.005$). Guanethidine, again, caused a pronounced decrease in ³H MA levels in both the particulate ($P < 0.001$) and supernatant ($P < 0.001$) fractions.

A combination of reserpine plus protriptyline caused an almost complete release of ³H MA both 15 min and 24 hrs after administration of the labelled amine. This combination demonstrated actions similar to but more potent than guanethidine in that it caused a loss from both fractions at short and long intervals after ³H MA administration.

Effect of guanethidine on the in vitro uptake of C-adrenaline by bovine adrenal medullary granules. Lower concentrations of guanethidine had a slight inhibiting effect on the ATP-Mg-dependent uptake of C-adrenaline into the adrenal medullary granules (Table III). In higher concentrations (5×10^{-3}M) however guanethidine caused a relatively pronounced blockade of the C-adrenaline uptake.

TABLE III Influence of guanethidine on the uptake of C-adrenaline by bovine adrenal granules

Drug Concentration (M)	Number of experiments	Percent Inhibition
$1 \times 10^{-[illegible]}$	2	4.5
$5 \times 10^{-[illegible]}$	1	14.0
$1 \times 10^{-[illegible]}$	4	28.5
$2.5 \times 10^{-[illegible]}$	1	38.6
$5 \times 10^{-[illegible]}$	4	70.5

The concentration of adrenaline (labelled plus unlabelled) in the incubation medium was $5 \times 10^{-[illegible]}$ M.

Discussion

The existence of two mechanisms for the concentration of catecholamines in adrenergic nerves, the transport through the nerve cell membrane and an uptake into amine-storing granules, is now generally accepted (Carlsson, Hillarp and Waldeck 1963 Lindmar and Muscholl 1964 Giachetti and Shore 1966) Guanethidine appears to be retained in adrenergic neurons in sites which may be identical to those storing noradrenaline (Chang, Costa and Brodie 1964) Both noradrenaline and reserpine reduce the uptake of guanethidine into sympathetically innervated tissue, and reserpine can release ³H-guanethidine already accumulated (Chang, Costa and Brodie 1965) Furthermore, guanethidine can be released by sympathetic nerve stimulation (Bouillin, Costa and Brodie 1966)

Evidence has accumulated which demonstrates that guanethidine can interfere with the catecholamine storage capacity of sympathetic nerves. Serveral authors have demonstrated the existence of a dual effect of guanethidine on adrenergic nerves on the nerve cell membrane and a second on an intracellular amine-concentrating ism (Lindmar and Muscholl 1964 Shore and Giachetti 1966)

Within 1/2 h after its administration guanethidine reduced the uptake of injected ³H-α Me NA by approximately 70 per cent. This early impairment was, in all probability primarily due to a membrane pump blocking effect rather than a granular blocking action, since the small amount of ³H-α Me-NA that was taken up was distributed almost normally This is supported by the finding that a drug which specifically blocks the uptake into adrenergic granules such as reserpine does markedly alter the subcellular distribution of injected ³H-α-Me NA even at this early time period (Carlsson *et al* 1967) After 12 hrs, however membrane pump blocking effect seems to be diminished since the total uptake is increased but a granular blocking action remains. Apparently guanethidine in the dose used can block both uptake mechanisms, but the onset of membrane pump blockade is more rapid while the granular blockade is more persistent These findings are in agreement with the *in vitro* studies of Shore and Giachetti (1966) which showed that the action of guanethidine on the membrane pump was more readily reversible than the effect on the intracellular storage mechanism.

In the present investigation ^{3}H MA was used in an attempt to further elucidate different mechanisms of drug-induced amine release. Metaraminol has the advantage of being resistant both to monoamine oxidase and catechol-O-methyl transferase, and appears to be a valuable tool in the investigation of adrenergic mechanisms. Guanethidine, but not reserpine was able to release ^{3}H MA from both the particulate and supernatant fractions 15 min as well as 24 hrs after administration of the labelled compound. Reserpine was without effect at the earlier interval, but reduced the ^{3}H MA content of the particulate fraction at the later period. Protriptyline had no effect on the particulate fraction at either time. Its only action was to reduce the concentration of ^{3}H MA in the supernatant fraction. From this data it is apparent that the membrane pump blocking action of guanethidine cannot be responsible for the decrease of ^{3}H MA in the particulate fraction since protriptyline, a potent membrane pump blocking drug, did not affect the particulate fraction. The effect seen on the particulate fraction is probably due to a reserpine like action of guanethidine. The total depletion seen after guanethidine appears to correspond more closely to a combined regimen of reserpine and protriptyline, i.e. simultaneous blockade of both membrane pump and granular-concentrating mechanisms. Our studies and those of Carlsson and Waldeck (1965) demonstrate that such a combination is very effective in releasing ^{3}H MA from the heart.

Earlier *in vitro* studies could not show an appreciable blocking effect of guanethidine on the uptake of amines into isolated adrenomedullary granules. Carlsson, Hillarp and Waldeck (1963) found that guanethidine in a concentration of $8 \times 10^{-}$[illegible] M caused only a 15 per cent inhibition of ^{14}C-adrenaline uptake into bovine adrenal granules, however a higher concentration was not tried. Furthermore, the *in vitro* uptake of ^{14}C-adrenaline into rabbit adrenal granules after the *in vivo* administration of guanethidine was not blocked (Lundborg, unpublished results). However, as can be seen from Table III, high concentrations of guanethidine ranging from $1 \times 10^{-}$[illegible] to 5×10^{-3} can inhibit the adrenal granular uptake mechanism. It is possible that in order for guanethidine to exert its granular blocking action *in vivo* quite high intracellular concentrations must be achieved. An active transport of guanethidine into adrenergic neurons could help achieve this condition. That guanethidine is actively transported into the cell is supported by the finding that blockade of the membrane pump greatly reduces the accumulation of ^{3}H-guanethidine (Brodie, Chang and Costa 1965). If the adrenal medullary cells lack an effective membrane pump then guanethidine would not be concentrated and therefore could not impair uptake mechanisms in this organ. Such a hypothesis could explain the lack of effect of guanethidine in causing adrenal catecholamine depletion *in vivo* as reported by Athos *et al.* (1962) and Cass and Callingham (1964).

Costa (1966) has concluded that adrenergic blockade and noradrenaline loss result from the same action of guanethidine, i.e. persistent change in the basic properties of adrenergic terminals. It was proposed that guanethidine produces a prolonged depolarization of presynaptic sympathetic nerve terminals and that this depolarization prevents the noradrenaline released from being returned to storage.

However the recent evidence of Cabrera, Torrance and Viveros (1966) make the conclusion unlikely. These authors, using electrophysiological techniques, showed that guanethidine, rather than causing a persistant depolarization, actually holds the nerve membrane polarized at, or even above, the resting level. Therefore an alternative explanation of the depleting action of guanethidine must be sought. The present results and those of Malmfors (1964) indicate that impairment of the membrane pump and/or granular uptake mechanisms can, under certain conditions, lead to amine depletion. Since guanethidine possesses both actions it is not remarkable that this drug can deplete catecholamines. The ability of guanethidine to block the membrane pump may also explain the potentiation of exogenously injected amines seen after guanethidine administration (Abboud, Ekstein and Wendling 1962).

Kuntzman *et al.* (1962) have suggested that reserpine and guanethidine-induced noradrenaline release are produced by different mechanism since reserpine administration is not accompanied by signs of sympathomimetic activity while guanethidine-caused loss is so accompanied. Our experiments and those of others (Lindmar and Muscholl 1964, Shore and Giachetti 1966) indicate that this difference may be at least partly explained by the additional blocking action of guanethidine on the membrane pump. Thus the noradrenaline release induced by guanethidine, but not reserpine, may cause sympathomimetic effects since re-uptake of the released amines cannot be accomplished by the membrane pump and thus are released in higher concentrations onto receptor sites.

The research reported in this manuscript has been supported by the Swedish State Medical Research Council (B 67-14X-155-03 A) and the Faculty of Medicine, University of Göteborg, Sweden. RES would like to thank West Virginia University for the leave of absence which allowed him to carry out this work, and the SSMRC for their financial support. The highly competent technical assistance of Miss Lena Ramstedt is gratefully acknowledged.

References

ABBOUD, F. J. EKSTEIN and M. WENDLING. Early potentiation of the vasoconstrictor action of norepinephrine by guanethidine. *Proc. Soc. exp. Biol. (N.Y.)* 1962. *110*: 489—492.
ATHOS, W. B. McH. OWEN, S. FIACCHINO and J. HILTON. The effects of guanethidine on the adrenal medulla. *J. Pharmacol. exp. Ther.* 1962. *137*: 229—234.
BOULLIN, D. E. COSTA and B. BRODIE. Discharge of tritium-labeled guanethidine by sympathetic nerve stimulation as evidence that guanethidine is a false transmitter. *Life Sci.* 1966. *5*: 803—808.
BRODIE, B. C. CHANG and E. COSTA. On the mechanism of action of guanethidine and bretylium. *Brit. J. Pharmacol.* 1965. *25*: 171—178.
CABRERA, R., R. TORRANCE and H. VIVEROS. The action of acetylcholine and other drugs upon the terminal parts of the postganglionic sympathetic fibre. *Brit. J. Pharmacol.* 1966. *27*: 51—63.
CARLSSON, A., Pharmacology of the sympathetic nervous system. In *Antihypertensive Therapy*. Ed. F. Gross. Springer-Verlag, Berlin 1966.
CARLSSON, A., N. A. HILLARP and B. WALDECK. Analysis of the Mg^{++}-ATP dependent storage mechanism in the amine granules of the adrenal medulla. *Acta physiol. scand.* 1963. *59*: Suppl. 215: 1—38.
CARLSSON, A., P. LUNDBORG, R. STITZEL and B. WALDECK. Uptake, storage and release of ^3H-α-methyl-noradrenaline. *J. Pharmacol. exp. Ther.* 1967. In press.

CARLSSON, A. and B. WALDECK, Rapid release of ^{3}H-metaraminol induced by combined treatment with protriptyline and reserpine. *J Pharm. Pharmacol.* 1965 *17* 327—328.

CASS, R. and B. CALLINGHAM, Some effects of drugs which influence sympathetic transmission on tissue catecholamine levels in the rat. *Biochem. Pharmacol.* 1964. *13* 1619—1625

CASS, R., R. KUNTZMAN and B. BRODIE, Norepinephrine depletion as possible mechanism of action of guanethidine (SU 5864) new hypotensive agent. *Proc Soc Exp. Biol.* (N Y) 1960. 103 871—872.

CASS, R. and T SPRIGGS, Tissue amine levels and sympathetic blockade after guanethidine and bretylium. *Brit. J Pharmacol.* 1961 *17* 442—450.

CHANG, C., E. COSTA and B BRODIE, Reserpine-induced release of drugs from sympathetic nerve endings. *Life Sci.* 1964 *3* 839—844

CHANG, C., E. COSTA and B. BRODIE, Interaction of guanethidine with adrenergic neurons. *J Pharmacol. Exp Ther* 1965. *147* 303—312.

COSTA, E., Interaction of guanethidine and bretylium with adrenergic neurones. In *Mechanisms of Release of Biogenic Amines.* Ed. U.S. von Euler S. Rosell and B. Uvnäs. Pergamon Press, Oxford 1966. 291—305

GIACHETTI, A. and P SHORE, Studies in vitro of amine ptake mechanisms in heart. *Biochem. Pharmacol* 1966. *15* 607—614.

HILLARP N A Isolation and some biochemical properties of the catecholamine granules in the cow adrenal medulla. *Acta physiol. scand* 1958. *43* 82—96.

KUNTZMAN R E. COSTA, G. GESSA and B. BRODIE, Reserpine and guanethidine action on peripheral stores of catecholamines. *Life Sci.* 1962. *1* 65—74

LINDMAR, R. and E. MUSCHOLL, Die Wirkung von Pharmaka auf die Elimination von Noradrenalin aus der Perfusionsflüssigkeit und die Noradrenalinaufnahme in das isolierte Herz. Naunyn-Schmiedeberg *Arch. exp Path. Pharmak.* 1964. *247* 469—492.

MALMFORS, T Release and depletion of the transmitter in adrenergic terminals produced by nerve impulses after the inhibition of noradrenaline synthesis or reabsorption. *Life Sci.* 1964 *3* 1397—1402.

SANAN S. and M. VOGT Effect of drugs on the noradrenaline content of brain and peripheral tissues and its significance. *Brit. J Pharmacol.* 1962. *18.* 109—127

SHEPPARD, H. and J ZIMMERMAN, Effect of guanethidine (SU-5864) on tissue catecholamines. *Pharmacologist* 1959 *1* 69.

SHORE, P and A. GIACHETTI, Dual actions of guanethidine on amine uptake mechanisms in adrenergic neurones *Biochem. Pharmacol.* 1966. *15* 899—903

STITZEL, R. E and P LUNDBORG, Effect of reserpine and monoamine oxidase inhibition on the uptake and subcellular distribution of ^{3}H-noradrenaline *Brit. J Pharmacol.* 1967 *29* 99—104.

Acta Physiol. scand. 1968. 72 108—114

From the Department of Physiological Chemistry University of Lund, Sweden

Comparison of Dipeptidase Activity in Different Tissues of the Pig

By

LARS JOSEFSSON OVE NORÉN and HANS SJÖSTRÖM

Received 17 May 1967

Abstract

JOSEFSSON L., O NORÉN and H SJÖSTRÖM. *Comparison of dipeptidase activity in different tissues of the pig* Acta physiol. scand. 1968. 72 108—114

A comparative investigation of three dipeptidase activities (L-alanyl L-glutamic acid L-alanyl-L-proline and glycyl-L-leucine dipeptidase activity) of the trachea, the tuba uterina, the uterus and the small intestine of the pig has been made to study if the intestinal dipeptidases differ in their characteristics from the dipeptidases of other tissues. No indication was obtained supporting the idea that the intestinal dipeptidases would be different and constitute a group of enzymes, particularly adapted for intestinal digestion

Recently various dipeptidase activities in the intestinal mucosa of different species have been thoroughly investigated in this laboratory (Josefsson and Lindberg 1965 a and b, and 1966 Josefsson and Sjöström 1966 Lindberg 1966 a From these studies the question arose whether these enzymes, which perform the final digestion of the nutrient proteins in the intestine differ in their characteristics from the dipeptidases present in other tissues and whether the intestinal mucosa contains relatively larger amounts of dipeptidases than tissues not adapted for digestion purposes.

Although a vast number of investigations during the last decades have demonstrated that the dipeptidase activities are generally distributed in most animal tissues (for ref see Lindberg 1966 b differences in the experimental method used in the various studies make any evaluation of such comparative question difficult. Therefore a comparative study of the dipeptidase activity in various tissues was made utilizing the spectrophotometric assay method (Josefsson and Lindberg 1966 a) The mucosa of tuba uterina trachea and small intestine was selected for the study Uterine muscle was also included as it was known to contain high dipeptidase activity (Smith 1948)

Materials and methods

Source of tissue Trachea, tuba uterina, uterus and small intestine from adult pigs were used. After slaughter the organs were immediately removed, put into ice and the various tissue extracts were prepared within 1—2 hrs of death. The organs were measured in respect to their length and weight.

Substrates. L-Alanyl-L-glutamic acid, L-alanyl-L-proline and glycyl-L-leucine of known purity (Josefsson and Lindberg 1965 a, and 1966) were used as substrates.

Amino acids. The amino acids were products of Mann Research Lab., New York, and previously tested for purity (Josefsson and Lindberg 1965 a, and 1966) They were used in the same concentrations and combinations as reported earlier (Josefsson and Lindberg 1965 a, and 1966)

Assays. The dipeptidase activities were determined by the spectrophotometric method previously described (Josefsson and Lindberg 1965 a, and 1966) Buffers and metal salt solutions were also the same as previously reported. pH of the digest mixtures was measured before the digestion (glass electrode) Units of dipeptidase activity were calculated according to previous definition (Josefsson and Lindberg 1965 b)

Preparation of enzyme solutio All procedures were performed at 4 Tissue extracts were prepared by scraping off the epithelium from the whole length of the trachea and the tuba uterina, and by using small pieces cut from the uterine body Based on their wet weight, the various tissues were added to 5 vol of 0.25 M sucrose solution, extracted for 24 hours and then centrifuged at 27,000 × *g* for 30 min (International, model HR 1) The clear supernatants were diluted with water according to their protein content and used directly as enzyme solutions

To study the release of the dipeptidase activities by the preceding procedure equivalent amounts of the various tissue preparations were weighed in two sets of separate vessels. 5 vol of 0.25 M sucrose solution were added to each vessel. After 2 hrs one set of the vessels were taken for centrifugation as above and the supernatants were removed and assayed for dipeptidase activity and for protein content. The sediments were washed in 5 ml of 0 25 M sucrose solution and homogenized for 2 min at 14,500 rpm (MSE-homogenizer) to release the residual activity (Josefsson and Sjostrom 1966) The homogenates were centrifuged as above and the supernatants were removed and assayed for dipeptidase activity and protein content After 23 hrs of extraction the second set of vessels was taken through the same procedures.

To analyze the various tissues in respect to their total dipeptidase activity the epithelium from whole tracheae and tubae uterinae was scraped off, added to 0 05 M sodium phosphate buffer pH 7.0 (5—10 vol) and homogenized for 2 min at 14,500 rpm The homogenates were centrifuged at 27,000 × *g* for 30 min. The supernatants were removed quantitatively and analyzed for their dipeptidase activity and protein content Intestinal mucosa (from 5 cm lengths of gut) taken 1 and 3 m distal of the pylorus of intestines of known lengths, and pieces cut from the body of different uteri of known weight, were also included in the experiments and analyzed in the same manner

Protein was determined according to the procedure of Lowry *et al.* (1951) using crystallized bovine ribonuclease (Armour Lot No DCO 850) for preparation of the standard curve

Results

Determination of pH optima

The different dipeptidase reactions were studied over a pH range from 5 5 to 9 by using enzyme preparations from the three different tissues. As shown in Fig 1—3 the shape of the curves was found to be very similar and independent of the source of the enzymes The pH optima showed only minor variation and were found to be closely the same as previously obtained in the studies of the intestinal reactions, i.e. 7 4 for L-alanyl-L-glutamic acid dipeptidase 7 7 for glycyl L leucine dipeptidase and 7.0 for L-alanyl L proline dipeptidase (Josefsson and Lindberg 1965 a)

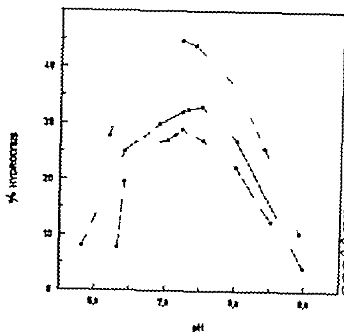


Fig. 1. Effect of pH on the L-alanyl-L-glutamic acid dipeptidase activity of different tissues of the pig
( ) Trachea.
(— — —) Tuba uterina.
(— —) Uterus.

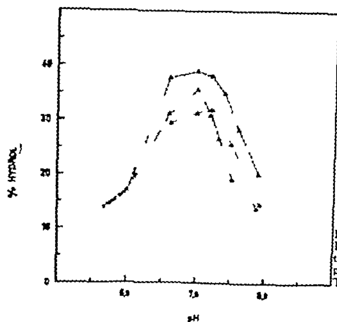


Fig. 2. Effect of pH on the L-alanyl L-proline dipeptidase activity of different tissues of the pig ( ) Trachea. (———) Tuba uterina. (— —) Uterus.

Effect of metal ions

As the dipeptidases are influenced by bivalent ions, the effect of Co^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+} ions on the different hydrolyses was studied, to determine whether the various tissue preparations showed any difference in their metal-ion specificity. The

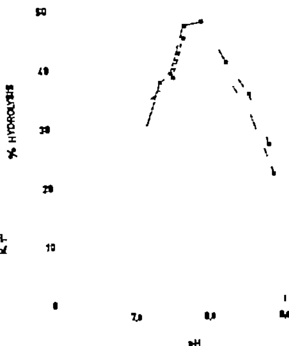


Fig. 3. Effect of pH on the glycyl-L-leucine dipeptidase activity of different tissues of the pig.
( ) Trachea.
(— — —) Tuba uterina.
(— —) Uterus.

metal ions were added to the assay mixtures with the buffer in 10 and 100 μM concentrations (Josefsson and Lindberg 1965 a) previously shown to be a suitable procedure. The results are given in Table I, compared with the rate of hydrolysis in the absence of added metal ions.

No significant differences were obtained between the three different enzyme preparations. The hydrolysis of L-alanyl L-glutamic acid was inhibited by Co^{2+} and Mn^{2+} ions but was unaffected by Mg^{2+} and Zn^{2+} ions when they were added with phosphate buffer. Zn^{2+} ions in borate buffer however inhibited the activity in agreement with our previous findings (Josefsson and Lindberg 1965 a). No effect of the metal ions was obtained on the L-alanyl L-proline dipeptidase activity of any of the tissue preparations. Co^{2+} and Mn^{2+} ions caused a similar degree of reduction of the glycyl L-leucine dipeptidase activity while Mg^{2+} ions showed no influence on this reaction in any of the assays. Depending on the buffer system used in the assay Zn^{2+} ions either increased (phosphate) or reduced (borate) the activity in accordance with our previous observation on the intestinal reaction (Josefsson and Lindberg 1965 a).

TABLE I Comparison of the influence of bivalent metal ions on dipeptidase activities in different pig tissues. Optimal pH and phosphate buffer

Substrate	Metal ion	Concentration (mμmoles)	Relative rate of hydrolysis[a]		
			Trachea	Tuba uterina	L[illegible]
L-Alanyl-L-glutamic acid	Co	1	0.6	0.7[a]	0.6
		10	0.4	0.2	0.3
	Mn	1	0.9	0.8	1.1
		10	0.6	0.7	0.7
	Mg	1	0.9	1.1	1.3
		10	0.9	1	1
	Zn^{++}	1	0.8	0.8	1
		10	0.8	0.5	1
L-Alanyl-L-proline	Co[b]	1	0.8	1	1
		10	1	1.1	1
	Mn	1	1	1	1
		10	1	1	1
	Mg	1	1	1	1
		10	1	1	1
	Zn^{++}	1	1	1	1
		10	0.8	1.1	1.1
Glycyl-L-leucine	Co[b]	1	0.8[a]	0.7[a]	1.1
		10	0.6	0.7	0.8
	Mn	1	0.8	0.7	1
		10	0.6	0.6	0.7
	Mg[b]	1	0.9	0.9	1
		10	0.9	0.9	1
	Zn[b]	1	0.9	0.8	1.4
		10	0.5	0.5	1.4

[a] The rate of hydrolysis without metal ions added taken as 1
[b] Borate buffer

Studies on the release of the dipeptidases

Previous studies had shown that the intestinal dipeptidases in contrast to the other intestinal hydrolases were rapidly released from the mucosa cells into the surrounding solvent (Josefsson and Sjöström 1966). To study if this behaviour was specific for the intestinal dipeptidases, the release of the dipeptidases of the other tissue cells was investigated. The results obtained (Table II) demonstrated that the dipeptidases of the epithelial cells of both the trachea and the tuba uterina were released as rapidly as those of the intestinal mucosa. The low yields obtained with uterine muscle may indicate that the dipeptidases are more firmly bound intracellularly in this tissue but it seems more likely that the results are due to poor penetration of solvent through the larger tissue masses.

TABLE II Release of dipeptidase activities from arious pig tissues into 0.25 M sucrose solution. N stirring,[1] 4

Substrate	Time (hours)	Released activity (of total)		
		Trachea	T ba uterina	Uterus
L-Alanyl-L-glutamic acid	2	84	98	59
	23	99	95	66
L-Alanyl-L-proline	2	84	88	48
	23	82	85	67
Glycyl-L-leucine	2	87	89	55
	23	99	95	65

Quantitative estimation f the dipeptidase activity

The figures obtained for the dipeptidase acti ities when related to the protein content of the investigated tissues are presented in Table III Only small variations were observed between the different tissues except for the jejunum-ileum region of the intestine the region with maximum activity (Josefsson and Lindberg 1965 b) which gave higher figures. When the total dipeptidase activity was calcu lated for each organ, the small intestine contained considerably large amounts, however due to its larger size It was also observed that the relative activ ty against th different dipeptides was approximately the same in the small intestine, the trachea and the uterus. These showed a relation between the glycyl-L-leucine dipeptidase, the L-alanyl-L-glutamic acid dipeptidase and the L-alanyl L-prolin dipeptidase of about 10 to 4 to 1 The tuba uterina differed in respect to ts L-alanyl L-proline dipeptidase content, having a relative amount only half of the other tissues.

TABLE III Specific activity of dipeptidases in various pig tissues

Substrate	Units of activity/mg protei				
	Small Intestine		Trachea	Tuba uterina	Uterus
	1 m distal pylorus	3 m distal pylorus			
L-Alanyl-L-glutamic acid	3.4	9 5	2.7	2 6	1 3
L-Alanyl-L-proline	0 98	2.4	0 91	0 35	0.31
Glycyl-L-leucine	8.4	24 7	7 6	7.2	3.6

Conclusion

The present results give no support for the idea that the intestinal dipeptidases constitute enzymes particularly adapted for intestinal digestive function. Thus, dipeptidases from the three investigated tissues showed the same pH optima and metal-ion specificity and these characteristics were the same as previously observed for intestinal dipeptidases (Josefsson and Lindberg 1965 a). Further the rapid liberation of the dipeptidases generally observed to occur in all the tissues under the present experimental conditions indicates that the dipeptidases of other tissues do not differ in their cellular attachment from those of intestinal mucosa. The present results therefore suggest that the intestinal dipeptidases accomplish the final breakdown of the nutrient proteins by means of physiologically adapted intestinal mucosa cells rather than by being specific in their own action. The larger amount of dipeptidases in the small intestine obtained when related to the protein content is also in agreement with this conclusion. A final answer must, however, await comparative studies on purified enzymes.

Miss I. Göransson and Mrs L. Rydberg are acknowledged for their skilful technical assistance. The investigation was supported by grants from the Swedish Medical Research Council (Project No 13X—17) and from the University of Lund.

References

Josefsson, L. and T. Lindberg, Intestinal dipeptidases I. Spectrophotometric determination and characterization of dipeptidase activity in pig intestinal mucosa. *Biochim. biophys. Acta* (Amst.) 1965 a. *105* 149—161.

Josefsson, L. and T. Lindberg, Intestinal dipeptidases II. Distribution of dipeptidases in the small intestine of the pig. *Biochim. biophys. Acta* (Amst.) 1965 b. *105* 162—166.

Josefsson, L. and T. Lindberg, Intestinal dipeptidases III. Characterization and determination of dipeptidase activity in adult rat intestinal mucosa. *Acta physiol. scand.* 1966. *66*. 410—418.

Josefsson, L. and H. Sjöström, Intestinal dipeptidases IV. Studies on the release and subcellular distribution of intestinal dipeptidases of the mucosa cells of the pig. *Acta physiol. scand.* 1966. *67*. 27—33.

Lindberg, T., Intestinal dipeptidases. Dipeptidase activity in the mucosa of the gastrointestinal tract of the adult human. *Acta physiol. scand.* 1966. *66*. 437—443.

Lindberg, T., Studies on intestinal dipeptidases. *Acta physiol. scand.* 1966 b. *69* Suppl. 285. 1—38.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the folin phenol reagent. *J. biol. Chem.* 1951. *193* 265—275.

Smith, E. L., Studies on dipeptidases II. Some properties of the glycyl-L-leucine dipeptidases of animal tissues. *J. biol. Chem.* 1948. *176* 9—19.

Acta physiol. scand. 1968. 72. 115—122

From the Department of Physiology, University of Göteborg, Sweden

The Effect of Reserpine on Transmission in the Spinal Cord

By

I. ENGBERG, A. LUNDBERG and R. W. RYALL[1]

Received 19 May 1967

Abstract

ENGBERG, I., A. LUNDBERG and R. W. RYALL. *The effect of reserpine on transmission in the spinal cord.* Acta physiol. scand. 1968. 72. 115—122.

In unanaesthetized acute spinal cats, in which monoamine oxidase is inhibited, an i.v. injection of reserpine gives a profound depression of transmission from the flexor reflex afferents (FRA) to primary afferents and motoneurones. The effect is similar to that evoked by the monoamine precursors DOPA or 5-HTP including a release of late reflex actions from the FRA. In chronic spinal cats reserpine has no effect on transmission from the FRA. The effect of reserpine is partially reversed by the 5-HT antagonist Deseril (which also partially reverses the effect of 5-HTP) but not by phenoxybenzamine (which reverses the effect of DOPA). It is postulated that reserpine acts by liberation of 5-HT from descending fibres. The possibility that reserpine also acts on transmission from the FRA through liberation of noradrenaline is discussed.

Histochemical investigations have focused the interest on monoaminergic synapses in the central nervous system. One of the tools employed in the analysis of central monoaminergic transmission is the intravenous injection of the monoaminergic precursors, dihydroxyphenylalanine (DOPA) or 5-hydroxytryptophan (5-HTP). The spinal cord is a particularly favourable preparation for analysis of the mechanism by which these precursors act because all monoaminergic nerve terminals belong to descending spinal pathways (Carlsson *et al.* 1964). An intravenous injection of DOPA or 5-HTP depresses transmission in some pathways from the flexor reflex afferents (FRA) in the spinal cord. The suggestion that DOPA acts by liberating noradrenaline from synaptic terminals (Andén *et al.* 1964, 1966b) was based on a detailed pharmacological analysis and it was postulated that there is a descending noradrenergic pathway with an inhibitory effect on transmission in some pathways from the FRA (Andén *et al.* 1966b).

The effect of 5-HTP has not been analyzed in such detail but the finding that it

[1] Fellow of IBRO-UNESCO and the Wellcome trust. Present address: Division of Anesthesiology, Montefiore Hospital and Medical Center, New York.

has no effect after inhibition of the decarboxylase (Jukes and Lundberg unpublished) shows that it acts by formation of 5-hydroxytryptamine within the spinal cord. Hence we must consider the possibility that there may exist also a 5-hydroxytryptaminergic pathway with inhibitory effect on transmission from the FRA.

For further analysis it would be desirable to liberate these monoamines by some process not involving the administration of their precursors, for then it would be possible to exclude that these precursors may be acted upon by enzymes located outside the monoaminergic nerve terminals (cf Anden *et al* 1966b) The most direct experiment stimulation of the descending pathways is difficult to perform because of the requirement for selective activation of the monoaminergic pathways (cf Lundberg 1966) Another possibility is to employ drugs which liberate the transmitters but are not precursors. Reserpine is the best known drug of this type. It blocks monoaminergic transmission by depleting the stores of transmitter The present investigation is, however not concerned with this blocking effect by reserpine but with its initial action of liberating the transmitter It has been postulated that reserpine acts primarily on the storage particles from which the monoamines are released into the axoplasm (Carlsson 1966) If the monoamine oxidase (MAO) is inhibited then the monoamines would be expected to accumulate in the axoplasm and leak out through the nerve membrane Andén *et al* (1964) found that injection of reserpine after MAO inhibition has an effect similar to that produced by DOPA and 5-HTP This finding is analyzed in the present paper A preliminary report has been given (Engberg Lundberg and Ryall 1966)

Methods

All 11 expts were made on unanesthetized anaemically decorticated cats (*cf* Andén *et al* 1966a) which were made spinal by transection of the cord in the lower thoracic region. The cats were immobilized with gallamine triethiodide (Flaxedil, *M & B.*) and artificially ventilated. The blood pressure was measured continuously all the experiments. The technique of conditioning monosynaptic test reflexes and for recording of dorsal root potentials has been described (Eccles and Lundberg 1959 Carpenter *et al.* 1963)

The following drugs were used methysergide (Deseril, *S d*) nialamide, phenoxybenzamine and reserpine (Serpasil, *CIBA*) They were injected i.

Abbreviations ABSm, anterior biceps-semimembranosus DOPA, L-3 4-dihydroxyphenylalanine DRP dorsal root potential FRA, flexor reflex afferents G-S gastrocnemius-soleus 5-HT,5-hydroxytryptamine 5-HTP 5-hydroxytryptophan J joint (posterior knee joint nerve) MAO monoamine oxidase NA, noradrenaline PBSt, posterior biceps-semitendinosus; SP superficial peroneal Sur sural.

Results

We have confirmed the finding that an injection of reserpine (3 mg kg) has no effect on transmission from the FRA when the MAO is not inhibited (Andén *et al* 1964)

Fig 1 illustrates the effect of an intravenous injection of reserpine on the DRPs in a cat, which had previously received the MAO inhibitor nialamide. The control records A—D were taken immediately before the injection of reserpine. E and F obtained after reserpine, show a marked inhibition of the DRP evoked by stimula-

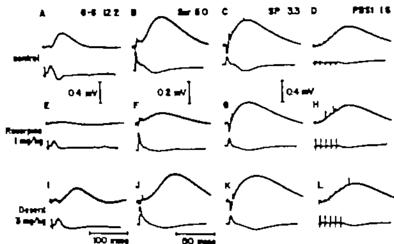


Fig. 1 The effect of reserpine on DRPs. Recording of DRPs (pper traces) was made from the most caudal dorsal rootlet in L6 and of the incoming olley (lower traces) from the L7 dorsal root entry zone. Stimulation of the G-S nerve in A, E and I of the Sur nerv in B, F and J of the SP nerv In C, G and K and of the PBSt nerv (short train) in D H and L. Stimulus strengths are given abov each column in multiples of threshold strengths for the nerves. The control records A—D were taken 180 min after the injection of nialamide (50 mg/kg) and immediately before the injection of reserpine. E—H were taken 37 min after the injection of reserpine (1 mg/kg) and I—L 63 min after reserpine and 10 min after an injection of Deseril 3 mg/kg. 0.2 mV calibration applies to DRPs in B F and J 0.4 mV to other DRPs. 100 msec time scale for A, E and I and 50 msec for the remaining records

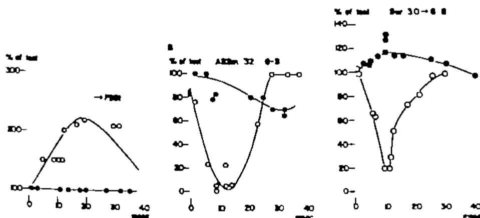


Fig 2 The effect of reserpine on the synaptic action evoked in motoneurones by olleys in the FRA. All curves show the effect of single conditioning olley on monosynaptic test reflexes. 100 % on the ordinate is the unconditioned est reflex and he abscissa gives the interval be tween conditioning and testing volley arriving in the spinal cord Control curves (O were obtained immediately before reserpine but about 3 hrs after an injection of nialamide. Curves ith filled circles ( ) were obtained after the injection of reserpine 1 mg/kg (30 min in A and B, 11 min in C) A and B are from the same experiment but C from another Conditioning stimulus strengths in B and C are given in multiples of threshold for the nerves the effect in A was evoked from high threshold joint afferents.

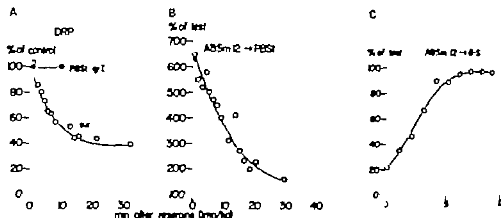


Fig. 3. Time course of the effect of an injection of reserpine on transmission from the FRA. Reserpine 1 mg/kg was injected about 3 hours after nialamide, 50 mg/kg. A shows the effect on the DRP evoked from cutaneous afferents (Sur ○) and the height of the DRP evoked by a train of group I volleys from PBSt is also shown for comparison. B shows the facilitatory effect of conditioning volley in high threshold muscle afferent on the monosynaptic test reflex from PBSt and C the corresponding inhibitory effect on the monosynaptic test reflex from G-S (conditioning-testing interval 19 msec in B and 4 msec in C). Conditioning stimulus strengths are given in multiples of threshold strength.

tion of the FRA. The DRP evoked from high threshold muscle afferents (E) was greatly depressed and the second component of the DRP evoked from the sural nerve (F) was also reduced in amplitude. There was much less change in the DRP evoked from the SP nerve (G). The latter DRP consisted mainly of component I, which is not evoked from the FRA, whereas the DRP evoked from the sural nerve in B largely consisted of component II, which is an FRA effect (Carpenter et al. 1963). The DRP in H evoked by a train of group I volleys in the nerve to PBSt was not significantly changed.

Similar effects were found on transmission from the FRA to motoneurones as investigated either by recording of ventral root discharges or by conditioning of monosynaptic reflexes. Fig. 2 A shows the effect of conditioning volleys in high threshold joint afferents on flexor monosynaptic test reflexes and B and C the effect of volleys in high threshold muscle afferents and cutaneous afferents respectively on extensor monosynaptic test reflexes.

The time course of the depression of the DRP from the sural nerve is shown in Fig. 3 A. Observe that the onset of the effect was very fast, usually there is noticeable effect within 1 min. A maximal action was obtained within 20 min like in curve B showing the effect on facilitation from the FRA. In curve C, from another experiment, the maximal effect was attained within 10 min. The effect of reserpine was longlasting; no recovery was seen after 4 hrs.

After DOPA or 5-HTP there are late reflex effects from the FRA: a late DRP and a late longlasting discharge in the ventral roots (Andén et al. 1966c, Jankowska et al. 1967). The same phenomena were also observed after reserpine.

In two control experiments the effect of nialamide alone on transmission from the FRA was followed for 4 and 5 hrs respectively. It changed neither the DRPs nor the facilitatory and inhibitory effect from the FRA on the monosynaptic test reflexes. The only change observed was the appearance of some resting activity in the ventral root but this may also occur spontaneously in unanesthetized spinal cats. Hence none of the effects described above were caused by nialamide.

All monoaminergic nerve terminals in the spinal cord belong to descending pathways (Carlsson *et al.* 1964). Hence if reserpine acts by liberating monoamines from synaptic terminals it would be expected to have no effect in chronic spinal cats. This was tested in three cats, two weeks after spinal transection in the lower thoracic region. The cats were pretreated with nialamide (50 mg/kg) for 2—3 hrs and it was found that reserpine in doses up to 3 mg/kg had no effect on the DRPs evoked from the FRA or on the excitatory and inhibitory effects of FRA stimulation upon monosynaptic test reflexes.

It seemed probable that reserpine acted in the acute spinal cat through liberation of NA or 5-HT or both of these monoamines. The next step was therefore to investigate whether antagonists of 5-HT and NA could reverse the effect of reserpine

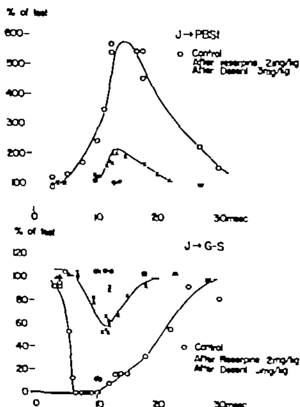


Fig. 4. The effect of Deseril on reflex depression caused by reserpine. The curves show the facilitatory effect of a single volley in high threshold joint afferents on the monosynaptic test reflexes from PBSt (upper graph) and the inhibitory effect upon the reflex from G-S (lower graph). The control curves were obtained 3.5 hours after the injection of nialamide and reserpine was then given. Deseril was given 105 min after reserpine. An injection of phenoxybenzamine (20 mg/kg) had been given 75 min after reserpine. The curves obtained after phenoxybenzamine were identical with the reserpine curves and are not shown in the graphs.

BOL and Deseril are 5-HT antagonists which partially reverse the effect of 5-HTP (Lundberg 1965). Deseril was given in 5 expts. after the reserpine effect had reached a steady state. In these experiments it was found that Deseril partially reversed the inhibitory action of reserpine on transmission from the FRA. This is illustrated in Fig. 1 I and J for the DRPs evoked from the FRA, and in Fig. 4 the facilitatory and inhibitory effects evoked by a conditioning volley in high threshold joint afferents on flexor and extensor monosynaptic test reflexes. In the experiment of Fig. 4 phenoxybenzamine had been given before Deseril (*cf.* legend). However, the effect of Deseril was also observed without a preceding injection of phenoxybenzamine. The effect in Fig. 1 was exceptionally large. In the concentration employed in these experiments Deseril in itself had no effect on transmission from the FRA to motoneurones but sometimes slightly prolonged the DRP. In two control experiments it was shown that Deseril does not reverse or otherwise influence the inhibitory effect of DOPA on transmission from the FRA. Phenoxybenzamine (20–25 mg/kg) was given after reserpine in 4 expts. This drug, which within a few minutes completely reverses the action of DOPA (Andén *et al.* 1966a), had no clear-cut blocking action on the effect of reserpine whether or not Deseril had been given before. In 2 expts. there was a weak antagonizing action with an onset some 20 min after the injection of phenoxybenzamine but this action is of extremely doubtful significance since phenoxybenzamine in these amounts lowered the blood pressure and large intravenous infusions of dextran had to be given to keep the arterial pressure at 80 mm Hg.

Discussion

It is well known that reserpine liberates the transmitter from peripheral and central monoaminergic nerve terminals (*cf.* Carlsson 1966). It is important to exclude that the effects produced by an injection of reserpine are not caused by circulatory changes evoked by noradrenaline liberated from peripheral sympathetic terminals. From this point of view it is relevant that an injection of reserpine had no effect on transmission from the FRA in chronic spinal cats investigated two weeks after transection of the spinal cord. Since reserpine increased the blood pressure to the same extent in acute and chronic spinal cats and therefore presumably had similar effect in the two preparations it is inferred that the effect in the acute spinal cat is produced centrally. A further reason for caution in using this drug as a tool in the analysis of central monoaminergic transmission is the difficulty in excluding that it may influence other synaptic mechanisms in the central nervous system. The finding that reserpine acts only after inhibition of the MAO strongly suggests that its inhibitory effect on transmission from the FRA is caused by liberation of monoamines. Furthermore since reserpine had no effect on transmission from the FRA in chronic spinal cats and all monoaminergic nerve terminals in the spinal cord belong to descending pathways (Carlsson *et al.* 1964), it seems overwhelmingly likely that the effect of reserpine in the acute spinal cat is caused by liberation of monoamines from the terminals of descending pathways. NA or 5-HT or both of these sub-

stances could be responsible since their precursors, DOPA and 5-HTP respectively have similar effects on transmission from the FRA (Andén *et al.* 1964, 1966a).

In order to analyze this problem further an attempt was made to reverse the effect of reserpine with substances known to block the effect of 5-HTP and DOPA. The finding that the effect of reserpine can be partially reversed by the 5-HT antagonist Deseril, suggests that at least part of the reserpine effect is caused by liberation of 5-HT. Hence these results support the hypothesis that there is a descending 5-hydroxytryptaminergic pathway with an inhibitory effect on transmission from the FRA. The reserpine effect is only to a small extent reversed by Deseril, but this substance is a rather ineffective 5-HT antagonist in the spinal cord as evidenced by the finding that it only partially reverses the effect of 5-HTP. Hence one cannot decide to what extent the effect of reserpine is caused by liberation of 5-HT.

Phenoxybenzamine, on the other hand, never gave a clear reversal of the action of reserpine on transmission from the FRA. This finding is difficult to interpret at present but it does not exclude the existence of a descending noradrenergic pathway with inhibitory effect on transmission from the FRA. Andén *et al.* (1964) originally assumed that phenoxybenzamine reversed the effect of DOPA by blocking adrenergic α-receptors. If this hypothesis is correct the explanation for the finding that phenoxybenzamine did not reverse the action of reserpine could be that the liberation of 5-HT dominates over that of NA (the effect of 5-HT liberation is only to a small extent reversed by the 5-HT antagonists). However Engberg and Ryall (1966) and Biscoe, Curtis and Ryall (1966) found that the effect of electrophoretically applied NA on interneurones could not be effectively antagonized by phenoxybenzamine. Engberg and Ryall postulated that phenoxybenzamine might reverse the effect of DOPA not through blockade of postsynaptic receptors but by an action on the presynaptic terminals. If this is the case phenoxybenzamine would not necessarily block the release of NA by reserpine or nerve impulses. Further investigations of the problem whether part of the reserpine effect on the spinal cord is caused by liberation of NA must await more knowledge of the NA receptors on which the DOPA effect is assumed to be exerted.

This work was supported by the Swedish Medical Research Council (Project No 14X-94-02A and 03B).

References

Andén, N. E., M. G. M. Jukes and A. Lundberg, Spinal reflexes and monoamine liberation. Nature (Lond.) 1964 202 1222–1223.

Andén, N. E., M. G. M. Jukes, A. Lundberg and L. Vyklický, The effect of DOPA on the spinal cord. 1. Influence on transmission from primary afferents. Acta physiol. scand. 1966a 67 373–386.

Andén, N. E., M. G. M. Jukes and A. Lundberg, The effect of DOPA on the spinal cord. 2. A pharmacological analysis. Acta physiol. scand. 1966b 67 387–397.

Andén, N. E., M. G. M. Jukes, A. Lundberg and L. Vyklický, The effect of DOPA on the spinal cord. 3. Depolarization evoked in the central terminals of ipsilateral Ia afferents by volleys in the flexor reflex afferents. Acta physiol. scand. 1966c 68 322–336.

Biscoe, T. J., D. R. Curtis and R. W. Ryall, An investigation of catecholamine receptors of spinal interneurones. Int. J. Neuropharmacol. 1966 5 429–434.

BOL and Deseril are 5-HT antagonists, which partially reverse the effect of 5-HTP (Lundberg 1965). Deseril was given in 5 expts. after the reserpine effect had reached a steady state. In these experiments it was found that Deseril partially reversed the inhibitory action of reserpine on transmission from the FRA. This is illustrated in Fig. 1 I and J for the DRPs evoked from the FRA and in Fig. 4 the facilitatory and inhibitory effects evoked by a conditioning volley in high threshold joint afferents on flexor and extensor monosynaptic test reflexes. In the experiment of Fig. 4 phenoxybenzamine had been given before Deseril (*cf.* legend). However, the effect of Deseril was also observed without a preceding injection of phenoxybenzamine. The effect in Fig. 1 was exceptionally large. In the concentration employed in these experiments Deseril in itself had no effect on transmission from the FRA to motoneurones but sometimes slightly prolonged the DRP. In two control experiments it was shown that Deseril does not reverse or otherwise influence the inhibitory effect of DOPA on transmission from the FRA. Phenoxybenzamine (20–25 mg/kg) was given after reserpine in 4 expts. This drug, which within a few minutes completely reverses the action of DOPA (Andén *et al.* 1966a) had no clear-cut blocking action on the effect of reserpine whether or not Deseril had been given before. In 2 expts. there was a weak antagonizing action with an onset some 20 min after the injection of phenoxybenzamine but this action is of extremely doubtful significance since phenoxybenzamine in these amounts lowered the blood pressure and large intravenous infusions of dextran had to be given to keep the arterial pressure at 80 mm Hg.

Discussion

It is well known that reserpine liberates the transmitter from peripheral and central monoaminergic nerve terminals (*cf.* Carlsson 1966). It is important to exclude that central effects produced by an injection of reserpine are not caused by circulatory changes evoked by noradrenaline liberated from peripheral sympathetic terminals. From this point of view it is relevant that an injection of reserpine had no effect on transmission from the FRA in chronic spinal cats investigated two weeks after transection of the spinal cord. Since reserpine increased the blood pressure to the same extent in acute and chronic spinal cats and therefore presumably had similar effect in the two preparations it is inferred that the effect in the acute spinal cat is produced centrally. A further reason for caution in using this drug as a tool in the analysis of central monoaminergic transmission is the difficulty in excluding that it may influence other synaptic mechanisms in the central nervous system. The finding that reserpine acts only after inhibition of the MAO strongly suggests that its inhibitory effect on transmission from the FRA is caused by liberation of monoamines. Furthermore since reserpine had no effect on transmission from the FRA in chronic spinal cats and all monoaminergic nerve terminals in the spinal cord belong to descending pathways (Carlsson *et al.* 1964) it seems overwhelmingly likely that the effect of reserpine in the acute spinal cat is caused by liberation of monoamines from the terminals of descending pathways. NA or 5-HT or both of these sub-

Acta physiol. scand. 1968. 72. 123—133

From the Department of Physiology University of Göteborg Sweden

Is the Tonic Decerebrate Inhibition of Reflex Paths Mediated by Monoaminergic Pathways?

By

I. Engberg, A. Lundberg and R. W. Ryall

Received 19 May 1967

Abstract

Engberg, I. A. Lundberg and R. W. Ryall. *Is the tonic decerebrate inhibition of reflex paths mediated by monoaminergic pathways?* Acta physiol. scand. 1968 72 123—133

In unanesthetized decerebrate cats the effect of monoamine blockers has been investigated on the decerebrate tonic inhibitory control of transmission from the FRA (flexor reflex afferents) to motoneurones. No certain effect is obtained after injection of phenoxybenzamine but 5-hydroxytryptamine antagonists (BOL or Deseril) gave a partial release from the tonic descending control of transmission from the FRA. The monoamine oxidase inhibitor nialamide, increased the effectiveness of the tonic control. The tonic decerebrate control may in part remain after complete destruction of the raphae nuclei. It is tentatively suggested that 5-hydroxytryptaminergic pathway descending from the raphae nuclei may contribute to, but probably is not solely responsible for the tonic decerebrate control of transmission from the FRA.

The decerebrate cat is characterized not only by rigidity but also by tonic inhibition of transmission from the flexor reflex afferents (FRA) to motoneurones, primary afferents and ascending pathways (Eccles and Lundberg 1959 Holmqvist and Lundberg 1961 Carpenter *et al* 1963 1965 Holmqvist *et al.* 1960). There is also inhibition of transmission from Ib afferents to motoneurones. It has recently been demonstrated that DOPA (1 3,4-dihydroxyphenylalanine) and 5-HTP (5-hydroxytryptophan) when injected [illegible] inhibit transmission of single reflexes in much the same way as occurs in the decerebrate state (Andén *et al.* 1964 1966a). These substances are intermediate metabolites in the synthesis of noradrenaline (NA) and 5-hydroxytryptamine (5-HT) and it is possible that they act by releasing transmitter from respectively noradrenergic or 5-hydroxytryptaminergic nerve terminals. This hypothesis is further supported by our findings that reserpine when injected

Fellow of IBRO-UNESCO and the Wellcome trust. Present address Division of Anesthesiology Montefiore Hospital and Medical Center New York

in an acute spinal cat, after inhibition of the monoamine oxidase, has a similar depressant effect on transmission from the FRA (Engberg *et al.* 1967). Reserpine has no such effect in the chronic spinal animal and Carlsson *et al.* (1964) have shown that all monoaminergic nerve terminals in the spinal cord belong to descending spinal pathways. So the question may be asked if the tonic inhibition in the decerebrate state is mediated by monoaminergic descending pathways.

We have now investigated the effect of antagonists of 5-HT and NA with respect to their ability to release spinal reflex pathways from the tonic decerebrate control and also the action of a monoamine oxidase inhibitor in enhancing this control. The effect of lesions in the brain stem region from which descending 5-hydroxytryptaminergic fibres originate has also been studied.

Methods

15 expts. were made on unanaesthetized cats decerebrated by intercollicular section. The cats were immobilized with gallamine triethiodide (Flaxedil, *M & B.*) and artificially ventilated. The blood pressure was measured continuously in 11 experiments and infusions of 6 % dextran given if the blood pressure fell below 80 mm Hg. The technique for conditioning monosynaptic reflexes and for recording of dorsal root potentials has been described (Eccles and Lundberg 1959, Carpenter *et al.* 1963). In some experiments electrolytic lesions were made in the brain stem. Details of this procedure are given in the legend of Fig. 6. The extent of the lesion was always investigated by serial sectioning of the brain stem after fixation in 10 % formalin and paraffin embedding. Every tenth section of 10 µ was examined with Nissl staining

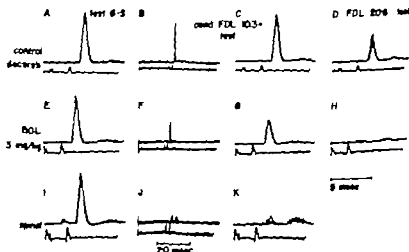


Fig. 1. Release from tonic inhibitory control of FRA inhibitory actions on G-S motoneurones produced by injection of BOL and by spinal transection. Upper traces: monosynaptic test reflexes evoked by double volleys in the G-S nerve and recorded in the S1 ventral root. Lower traces are from the dorsal root entry zone. Left column shows the unconditioned test responses, columns under C and D the effect of conditioning volleys in the FDL nerve at the strengths shown in column under B. A-D were obtained before and E-H after an i.v. injection of BOL (3 mg/kg). I-K were obtained 10 min after transection of the spinal cord in the lower thoracic region. Conditioning stimulus strengths are given in multiples of threshold for the FDL nerve. B, F and J were taken at the lower sweep speed shown under J, all other records at the faster speed shown under H. All records consist of 3—5 superimposed traces.

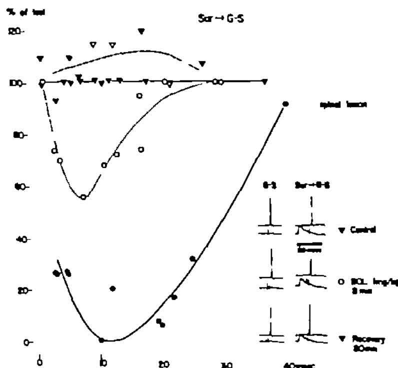


Fig 2. Effect of BOL on tonic decerebrate control. The ordinate gives the amplitude of conditioned test responses in per cent of the unconditioned test amplitude. The abscissa gives the time interval between conditioning (sural nerve) and testing (G-S nerve) volleys. The curves were obtained as indicated by inset records before (▽), 8 min after BOL (and 60 min after BOL (▼). The curve with filled circles (●) was obtained after transection of the spinal cord in the lower thoracic region.

The following drugs were used: bromolysergic acid diethylamide (BOL-148 *Sandoz*), 5-hydroxytryptophan, methysergide (Deseril, *Sandoz*), maleimide and phenoxybenzamine. All substances were injected.

Abbreviations: ABSm, anterior biceps-semimembranosus; BOL, bromolysergic acid diethylamide; DOPA, 1 3 4-dihydroxyphenylalanine; DRP, dorsal root potential; FDL, flexor digitorum longus; FRA, flexor reflex afferents; G-S, gastrocnemius-soleus; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; MAO, monoamine oxidase; PBSt, posterior biceps-semitendinosus; Sur, sural.

Results

The effect of phenoxybenzamine (25 mg/kg) on the decerebrate tonic control of transmission from the FRA to motoneurones was tested in 5 expts. In 3 of them it had no effect. In the remaining 2 expts. there was some release but this action was slow in onset and was probably caused by the marked fall of blood pressure after the drug injection in those experiments.

The 5-HT antagonists, BOL and Deseril, had a more clear-cut action. In the experiment of Fig. 1 the effect of conditioning volleys in high threshold muscle

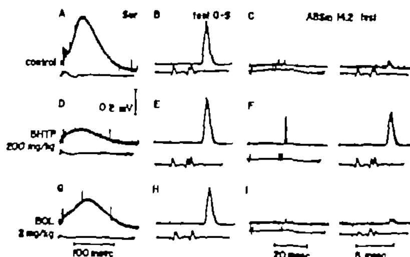


Fig. 3 Effect of BOL on the inhibitory action of 5-HTP. Upper traces in A, D and G are DRPs recorded from the most caudal dorsal rootlet in L6. Upper traces in all other records show monosynaptic test reflexes from G-S recorded in the S1 ventral root. Lower traces in all records are from the L7 dorsal root entry zone. Column under B shows the unconditioned test reflexes. Column under C shows the effect of a conditioning volley with conditioning stimulus strength indicated in multiples of threshold for the nerve. In C, F and I there are simultaneous records at two sweep speeds. Records in the left column show the conditioning-testing intervals. Records A–C were obtained shortly before an injection of 5-HTP (200 mg/kg). D–F were recorded 20 min after this injection. BOL was then given immediately and 10 min later records G–I were taken. Single traces in A, D and G, superimposed traces in all other records. 5 msec calibration also valid for B, E and H.

afferents on an extensor monosynaptic test reflex is investigated in a decerebrate cat before (B–D) and after (F–H) the injection of BOL and also after transection of the spinal cord (I–K). Two conditioning stimulus strengths were used. At the weaker strength there was very little effect before (B, C) but a marked effect after injection of BOL (F, G). The stronger conditioning volley gives a complete inhibition after BOL (H). Records I–K were taken after transection of the spinal cord and now the weaker of the two stimuli gave a maximal inhibition showing that BOL did not give a complete release of reflex transmission from the decerebrate control. The same phenomenon is illustrated in the curves of Fig. 2. Before BOL, a volley in cutaneous afferents gave a slight facilitation of the G-S test reflex from the sural nerve as commonly seen in the decerebrate state; after BOL there was a clear inhibition. An interesting feature is that the release from the tonic control is reversible as shown by the curve obtained 80 min after injection of BOL. In several experiments it was found that recovery from the effect of BOL occurred in 60–90 min. Hence it can be concluded that the action of BOL was not caused by a progressive deterioration in the state of the preparation. The lowermost curve in Fig. 2, obtained after transection of the spinal cord, again illustrates that BOL is able to release the reflex pathway from the descending decerebrate control to a small extent only. BOL gave release of transmission from the FRA to motoneurones in 4

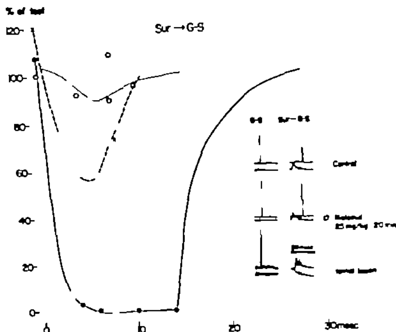


Fig 4 Depression of reflex transmission from FRA to G-S motoneurones produced by an injection of nialamide in the decerebrate cat. Curves were obtained as described for Fig — There was some initial release from the decerebrate tonic control as indicated by the inhibitory effect in the control curv (×) After nialamide the inhibitory action of conditioning olley in the Sur nerv decreased (○) Curve with filled circles (●) show the effect of the same conditioning olley after transection of the spinal cord in the lower thoracic region.

expts. out of 5 The most marked effect was on the inhibitory pathway to extensor motoneurones but slight effects were observed in some experiments on the excitatory pathway to flexor motoneurones and on the pathway to primary afferents (tested on the DRP evoked from the FRA) Similar effects were obtained w th deseril (3 mg/kg)

For comparison Fig 3 shows how BOL acted on the reflex depression that is seen after an injection of 5-HTP in the spinal preparation. After 5-HTP there was depression of the second component of the DRP (D) and also a marked decrease in the inhibitory action on a monosynaptic extensor test reflex (F) After BOL there was a reversal to the state before 5-HTP The effect on the DRPs in Fig 3 was unusually large.

If the decerebrate inhibitory control of transmission from the FRA depends on a 5-hydroxytryptaminergic pathway t may be expected that inhibition of the monoamine oxidase should enhance this tonic control. Fig 4 illustrates that this is the case. There was in this preparation partial release of the inhibitory pathway from cutaneous afferents to the G-S motor nucleus. An injection of nialamide markedly decreased this release

placed along the midline from the obex to a region 10 mm rostrally where a transverse lesion was made (*cf.* description in legend). The sample histological sections show that the midline structure was effectively destroyed by the lesion. Inspection of serial sections revealed a continuous destruction and only in very occasional places parts of the raphe nuclei escaped complete destruction. Presumably the lesion was complete from a functional point of view. The curves show that nevertheless some of the decerebrate control remained. After a complete transection at obex (the level of the most caudal electrolytic lesion) there was some additional release of inhibition and a very marked release of facilitation. In another experiment a larger lesion was made involving not only a complete destruction of the raphae nuclei but also the neighbouring medial reticular formation. Although this gave a complete release of inhibition from the FRA, some depression still remained of the excitatory pathway from these afferents to flexor motoneurones. These experiments show that the decerebrate inhibitory control of transmission from the FRA is not maintained exclusively from the raphe nuclei. This is the only conclusion that can be drawn from these experiments with lesions. It is, for example, not possible to state that the release caused by the destruction of the raphae nuclei indicates that these nuclei normally are responsible for part of the decerebrate control of reflex transmission; the release that occurred may have been caused by a coincident damage to the medial reticular formation.

Discussion

The experiments with injection of phenoxybenzamine never gave convincing evidence that this substance could release the reflex pathways from the tonic decerebrate inhibitory control. The evidence for the existence of a noradrenergic descending pathway with inhibitory effect of transmission in short latency pathways from the FRA is indirect but reasonably good (*cf.* Andén *et al.* 1966b). The explanation of the present findings may be that the noradrenergic pathway is not tonically active in the decerebrate state. However, although phenoxybenzamine effectively reverses the action of DOPA, doubts have been raised as to whether this effect is caused by a blockade of the postsynaptic receptors (Engberg and Ryall 1966). Hence the lack of effect of phenoxybenzamine is at present difficult to interpret as already discussed in relation to the experiments on the effect of reserpine (Engberg *et al.* 1967).

The finding that a 5-HT antagonist (BOL or Deseril) can give some release of reflex transmission from the tonic decerebrate control (and likewise that an inhibitor of MAO can increase this tonic control) suggests that this inhibition at least partly is maintained by activity in a descending 5-hydroxytryptaminergic pathway. The postulate of the existence of such a pathway is based not only on the effect of 5-HTP (Andén *et al.* 1964a) but also on the results from experiments with 5-HT antagonists after reserpine (Engberg *et al.* 1967). Another explanation of the results could be that the activity in the descending pathway maintaining the tonic control depends

on synaptic activation, at supraspinal levels, from 5-hydroxytryptaminergic pathways. Finally there is the possibility that the 5-HT antagonists can have an unspecific action decreasing the tonic activity in the centres maintaining the descending impulse flow. This may appear unlikely in view of the fact that the MAO inhibitor has the opposite effect.

The present results are of particular interest in relation to a previous suggestion that the raphae nuclei may be concerned with decerebrate control of transmission from the FRA (Lundberg 1964 p. 220). The reasons for this suggestion were the following. The tonic inhibitory control originates from centres located in the medial part of the lower brain stem (Holmqvist and Lundberg 1961). In the spinal cord the descending pathway is located in the dorsal part of the lateral funiculi (Holmqvist and Lundberg 1959). Anatomical investigations have not revealed any reticulospinal pathway with this location whereas it is probable that the axons of some of the raphe nuclei descend in this region of the spinal cord (Brodal, Taber and Walberg 1960). Since then, histochemical investigations have revealed that the raphe nuclei contain 5-HT neurones (practically all cell bodies in them give an intense 5-HT fluorescence after pretreatment with an MAO inhibitor) and that all descending axons of 5-HT neurones originate from the raphe nuclei (Dahlström and Fuxe 1965). However, it has now been shown that several pathways with inhibitory effect on transmission from the FRA descend from the caudal brain stem (*cf.* Lundberg 1966). In the present context the most interesting one is the dorsal reticulospinal system (Engberg *et al.* 1965, 1968), which produces the same effect on transmission from the FRA (and Ib afferents) as the tonic decerebrate control and whose axons descend in the dorsal part of the lateral funiculi (it is not known why this tract has not been identified anatomically). This dorsal reticulospinal system cannot be monoaminergic since the axons conduct at about 30m/sec, whereas the descending monoaminergic axons are unmyelinated with a diameter of about 0.5—1 μ (Dahlström and Fuxe 1965. Fuxe, personal communication). When the tonic control remains after destruction of the raphe nuclei (Fig. 6) it is probably mediated by the dorsal reticulospinal tract.

One possibility to differentiate between the activity in the dorsal reticulospinal system and the monoaminergic pathways lies with the late reflex actions from the FRA, which are released when the early reflex pathway is depressed by the monoamine precursors, DOPA and 5-HTP (Andén *et al.* 1964b, 1966a, c, Jankowska *et al.* 1967). These late reflex actions are not released when the pathways transmitting the early FRA actions are inhibited by activity in the dorsal reticulospinal system (Engberg *et al.* 1968). Furthermore, since in the decerebrate state these late reflex actions may be absent despite an effective descending inhibition of the early reflex actions it appears that there exists some other descending system inhibiting the early transmission from the FRA. Presumably this is the dorsal reticulospinal system described by Engberg *et al.* (1965, 1968). However in some decerebrate cats volleys in the FRA evoke late actions of the type that would be expected if the descending control was maintained by monoaminergic pathways. At present great

caution must be exercised in judging these findings, because we do not know yet if in this preparation these late actions are transmitted by spinal reflex pathways (as is the case after DOPA or 5-HTP) or whether they represent activity in a spino-bulbo-spinal pathway (*cf* Shimamura and Livingston 1963) If the former alternative proves to be correct it could be *that in* these preparations the descending control is dominated by the 5-hydroxytryptaminergic pathway and that the dorsal reticulospinal system is less active. We arrive at the tentative suggestion that both these pathways may contribute to the decerebrate tonic inhibition of transmission from the FRA.

We are indebted to Dr David Carpenter for his collaboration in a preliminary series of experiments concerning the effect of brain stem lesions on decerebrate reflex control.
This work was supported by the Swedish Medical Research Council (Project No 14X-94-02A and 03B)

References

Andén, N E, M. G. M. Jukes and A. Lundberg Spinal reflexes and monoamine liberation *Nature* (Lond.) 1964 *202* 1222—1223
Andén, N E., M G M. Jukes, A. Lundberg and L. Vyklický A new spinal flexor reflex *Nature* (Lond.) 1964b. *204* 1344—1345.
Andén, N E., M G M Jukes, A Lundberg and L. Vyklický The effect of DOPA on the spinal cord. 1 Influence on transmission from primary afferents. *Acta physiol. scand.* 1966a *67* 373—386
Andén, N E. M G M Jukes and A Lundberg, The effect of DOPA on the spinal cord 2 A pharmacological analysis *Acta physiol scand* 1966b *67* 387—397
Andén N E M G M Jukes, A Lundberg and L V Vyklický The effect of DOPA on the spinal cord 3 Depolarization evoked in the central terminals of ipsilateral I afferents by volleys in the flexor reflex afferents *Acta physiol. scand* 1966c. *68* 322—336
Brodal A E Taber and F Walberg The raphe nuclei of the brain stem in the cat II. Efferent connections *J comp Neurol.* 1960 *114* 239—259
Carlsson A B Falck, K Fuxe and N A. Hillarp Cellular localization of monoamines in the spinal cord. *Acta physiol scand.* 1964 *60* 112—119
Carpenter, D I Engberg, H Funkenstein and A Lundberg Decerebrate control of reflexes to primary afferents *Acta physiol scand* 1963 *59* 424—437
Carpenter, D I Engberg and A Lundberg, Differential supraspinal control of inhibitory and excitatory actions from the FRA to ascending spinal pathways *Acta physiol. scand* 1965 *63* 103—110
Dahlström, A and K Fuxe. Evidence for the existence of monoamine neurons in the central nervous system II Experimentally induced changes in the intraneuronal amine levels of bulbospinal neuron systems *Acta physiol scand* 1965 *64* Suppl. 247 7—36.
Eccles, R M and A Lundberg, Supraspinal control of interneurones mediating spinal reflexes *J Physiol.* (Lond) 1959 *147* 565—584
Engberg, I A Lundberg and R W Ryall, Reticulospinal inhibition of transmission through interneurones of spinal reflex pathways *Experientia* (Basel) 1965. *21* 612.
Engberg, I A Lundberg and R W Ryall, The effect of reserpine on transmission in the spinal cord. *Acta physiol scand* 1968. *72* 115—122.
Engberg, I A Lundberg and R W Ryall, Reticulospinal inhibition of transmission in reflex pathways. *J Physiol.* (Lond) 1968 In press.
Engberg I and R W Ryall The inhibitory action of noradrenaline and other monoamines on spinal neurones. *J Physiol* (Lond) 1966 *185* 298—322.
Holmqvist B and A. Lundberg, On the organization of the supraspinal inhibitory control of interneurones of various spinal reflexes *Arch ital Biol.* 1959 *97* 340—356.
Holmqvist B and A. Lundberg, Differential supraspinal control of synaptic actions evoked by volleys in the flexion reflex afferents in alpha motoneurones *Acta physiol. scand.* 1961 *54* Suppl. 186. 1—51

HOLMQVIST, B., A. LUNDBERG and O. OSCARSSON, Supraspinal inhibitory control of transmission to three ascending pathways influenced by the flexion reflex afferents. *Arch. ital. Biol.* 1960 *98* 60—80.

JANKOWSKA, E., M. G. M. JUKES, S. LUND and A. LUNDBERG, The effect of DOPA on the spinal cord. 5. Reciprocal organization of pathways transmitting excitatory action to alpha motoneurones of flexors and extensors. *Acta physiol. scand.* 1967 *70* 369—388.

LUNDBERG, A., Discussion in *Progress in Brain Research*. 1964 *12* 220. Elsevier Publishing Company Amsterdam.

LUNDBERG, A., Integration in the reflex pathway. In Nobel Symposium I. *Muscular Afferent and Motor Control*. 1966. 275—305. Almqvist & Wiksell. Stockholm.

SHIMAMURA, M. and R. B. LIVINGSTON, Longitudinal conduction systems serving spinal and brain stem coordination. *J. Neurophysiol.* 1963 *26* 258— 72.

Acta physiol. scand. 1968. 72. 134—138

From the Department of Pharmacology University of Göteborg, Sweden

Effect of Reserpine on the Urinary Excretion and the Tissue Levels of Noradrenaline in the Rat[1]

By

NILS-ERIK ANDÉN and MATTS HENNING

Received 23 May 1967

Abstract

ANDÉN, N. E. and M. HENNING. *Effect of reserpine on the urinary excretion and the tissue levels of noradrenaline in the rat.* Acta physiol. scand. 1968. 72. 134—138.

Reserpine (10 mg/kg i.p.) caused a reduction to about 10 per cent of the normal in the amount of noradrenaline excreted in the urine by adreno-demedullated rats. After 48 hrs the noradrenaline excretion had increased significantly (P<0.005) to about 50 per cent and after 72 hrs to about 55 per cent of normal. Simultaneously with these increases the sympathetic nerve function is restored partially and completely respectively. The noradrenaline levels of sympathetically innervated organs were reduced almost completely after the reserpine injection and there was no significant change of them at the time of the functional recovery. Similar results were obtained when an additional dose of reserpine (1 mg/kg i.p.) was given 72 hrs after the first one.

It is well known that reserpine depletes the noradrenaline (NA) stores of sympathetically innervated organs, blocks the transmission from the postganglionic sympathetic neurons and inhibits the nerve impulse induced release of NA from these nerves to the blood (Bertler, Carlsson and Rosengren 1956, Carlsson *et al.* 1957, Muscholl and Vogt 1958, Bertler *et al.* 1958). Furthermore the excretion of NA in the urine is reduced after reserpine treatment in animals (Carlsson *et al.* 1957, Leduc 1961, Johnsson 1963, Biscardi, Carpi and Oriangher 1964) as well as in man (Gaddum, Krivoy and Laverty 1958, Carlsson, Boje Rasmussen and Kristjansen 1959). This finding strengthens the concept of Euler (1956) that the postganglionic sympathetic neurons are the main source of the urinary NA. In the present study the time courses of the urinary NA excretion and of the tissue NA concentrations after a large dose of reserpine were investigated in the rat. To exclude any contribution of NA from the adrenal medulla the animals were bilaterally adreno-demedullated.

[1] Presented in part at the XIIth Scandinavian Congress in Physiology, Turku, August 24, 1966.

Material and methods

Adult, male, hooded rats weighing 250—350 g were used. At least 14 days prior to the urine collection bilateral adreno-demedullation was performed under pentobarbital-sodium anesthesia (Nembutal® 30—40 mg/kg) by enucleation according to Farris and Griffith (1949). During the first week after the operation the animals had access to sodium chloride solution (0.9 %) in addition to tap water. Collection of urine proceeded essentially as described by Andén (1965). Control determinations of the adrenaline (A) and NA excretion in the pooled urine from the same groups of three rat were performed on two different occasions before reserpine treatment and the mean value was set to 100 per cent. EDTA (disodium ethylenediamine tetraacetate 10 %, 0.2 ml per 10 ml urine) and ascorbic acid (2 %, 0.1 ml per 10 ml urine) were added to the urine. After paper filtration the specimens were neutralized to a pH of about 7 (indicator paper) with 5 N K_2CO_3 and allowed to stand for 10 min at 0 C. Following centrifugation at 10,000 × g for 10 min the amines were isolated from the supernatant by ion exchange chromatography. Differential estimation of NA and A in the sample was performed spectrophotofluorometrically according to the trihydroxyindole procedure (Bertler Carlsson and Rosengren 1958). The overall recovery was found to be 80—110 %. This was determined at frequent intervals by adding 0.5—1 µg NA and A to aliquots of urine sample. The NA contents of heart, spleen, femoral muscle and brain were determined according to Bertler Carlsson and Rosengren (1958). At least one week after the collection of the control urine the animals were injected with reserpine (Serpasil®) 10 mg/kg intraperitoneally and the urine was collected for 6-hour periods t various intervals after the injection. Care was taken to prevent hypothermia.

Results

The results are given in Fig 1. Before the reserpine treatment the adreno-demedullated rats excreted mainly NA in the urine (0.29 µg/kg hour s.e.m. = 0.024 n = 24) and only small amounts of A (0.012 µg/kg hour s.e.m. = 0.004 n = 24). Following the large dose of reserpine (10 mg/kg i.p.) the NA excretion was greatly reduced to a minimum of 12.5 per cent of the control values after 24 hrs. After 48 hrs the NA excretion had increased to 32.3 per cent of normal. This value is significantly ($p<0.005$) higher than that after 24 hrs. The amount of A in the urine showed a considerable increase for the first 24 hrs after the reserpine injection (maximum at 6—12 hrs 0.057 µg/kg hour s.e.m. = 0.029 n = 7) i.e. the urine contained about the same amount of A and NA at this time. To exclude the possibility that the reduction in NA excretion might have been influenced by the simultaneous increase in A excretion, an additional but smaller dose of reserpine (1 mg/kg i.p.) was given 72 hrs after the first injection to some animals. Thereafter the urinary catecholamine excretion was again examined at various intervals (Fig 1). The NA excretion, which after 72 hrs had reached about 55 per cent of the control values was lowered to about 24 per cent after 24 hrs but in this case there was a less pronounced increase in the A excretion. Forty-eight hrs after the injection of reserpine 1 mg/kg there was again a significant increase ($p < 0.05$) to about 46 per cent in the amount of NA excreted. The recovery of NA excretion in the urine seemed to be slow since normal values were not reached as late as 144 hrs after the smaller dose of reserpine.

After the urine collection the rats were sacrificed and the NA of the brain, heart, spleen and skeletal muscle was examined. Adreno-demedullated rats served as controls for the amine levels. In all organs examined there was a severe depletion of NA (less than 5 per cent of the control values) at 6 hrs after the first dose of reserpine (Fig 1). The NA content of the peripheral tissues remained largely

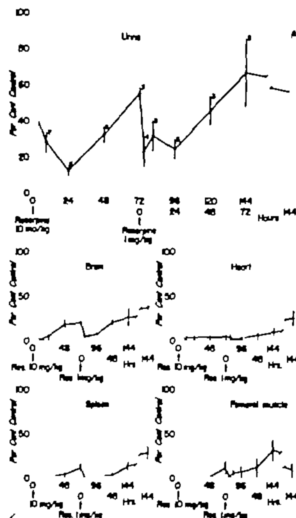


Fig 1 Effect of reserpine on the noradrenaline excretion in the urine and on the noradrenaline levels in the brain, heart, spleen and femoral muscle of adreno-demedullated rats. The values are means ± s.e.m. (number of urine determinations indicated by the small figures and number of tissue determinations was usually 5—6)
Normal noradrenaline concentrations: brain 0.40 μg/g (s.e.m. = 0.025, n = 6) heart 0.58 μg/g (s.e.m. = 0.024 n = 6) spleen 0.50 μg/g (s.e.m. = 0.060 n = 6) and femoral muscle 0.08 μg/g (s.e.m. = 0.008 n = 6)

changed after 48 hrs when the NA excretion in the urine increased significantly. After 72 hrs there seemed to be a certain increase in the NA content of the spleen and femoral muscle but it was not significant for any peripheral tissue. In the brain, however the NA content had increased somewhat ($p < 0.05$) at 48 and 72 hrs after reserpine 10 mg/kg as well as after 1 mg/kg. The same phenomenon was also observed for the content of dopamine in the brain (data not shown)

Discussion

After a large dose of reserpine it has been observed that the adrenergic transmission is partially returned 48 hrs and completely returned 72 hrs after administration of the drug. On the other hand the NA concentrations in the sympathetically innervated organs are lowered for a much longer time (Andén, Magnusson and Waldeck 1964 Andén and Henning 1966). In fact it is difficult to detect any NA

in the tissues when the nerve function recovers but there is a tendency to an increase of the NA at the time of complete functional return (see Results). The restoration of the sympathetic nerve function after reserpine treatment may depend on the concomitant and partial return of the ability of the sympathetic nerves to take up and retain tracer amounts of NA from the blood (Andén *et al.* 1964, Stjärne 1964, Carlsson 1965, Andén and Henning 1966, Iversen, Glowinsky and Axelrod 1965). It is to be noted that the uptake blockade by reserpine is restricted to the granules while the uptake through the cell membrane seems to be unaffected (Carlsson, Hillarp and Waldeck 1963, Lindmar and Muscholl 1964, Hillarp and Malmfors 1964, Carlsson and Waldeck 1967). From these data it appears that the adrenergic transmission is intact when a small but functionally important pool of the storage granules has the ability to incorporate NA.

In the present investigation it was observed that the urinary excretion of NA is markedly reduced as long as the pharmacological effects of reserpine are present. The NA excretion significantly increases simultaneously with the recovery of the sympathetic nerve function. On the other hand, the tissue levels of NA are still very low at the time of the functional restoration in agreement with previous findings.

The urinary excretion of NA increases only to about half of the normal values, when the recovery of the sympathetic nerve function is virtually complete. If the urinary excretion of NA exactly mirrors the NA release from the sympathetic nerves and if the inactivation of the released NA is unchanged, a postsynaptic mechanism in the adrenergic transmission can explain the discrepancy between the urinary excretion and the nerve function. Reserpine can produce a supersensitivity to injected NA but this phenomenon has only been observed after repeated injections of reserpine (Trendelenburg 1963). In previous investigations where tracer amounts of NA were administered there were no signs of supersensitivity to NA (Andén, Magnusson and Waldeck 1964, Andén and Henning 1966).

There is, however, the possibility that the adrenergic transmission is intact even when the amount of transmitter reaching the receptors is reduced moderately in an analogous way to that seen at the neuromuscular junction. Kuffler (1942) found that after treatment with small doses of curare the endplate potentials could be reduced but action potentials still occurred although after a longer latency. Furthermore, Elmqvist *et al.* (1964) observed that the amplitude of the endplate potentials in the intercostal muscles of patients with myasthenia gravis always were considerably reduced without necessarily producing a blockade of the neuromuscular transmission. Obviously there is a safety factor in the normal, cholinergic neuromuscular transmission and the same may be true for the adrenergic transmission.

This work has been supported by research grants from the Swedish Medical Research Council (K67 14X 302-03), from the Medical Faculty, University of Göteborg and from Läkemedelsindustriföreningen, Stockholm. For generous supply of reserpine (Serpasil®) we thank the Swedish CIBA, Stockholm. The skilful technical assistance of Miss Inger Pettersson is gratefully acknowledged.

References

ANDÉN N. E., The urinary excretion and the tissue uptake of noradrenaline after severe tissue depletion of noradrenaline by α-methyl-meta-tyrosine and metaraminol. *Acta physiol. scand.* 1965 *64* 136—145

ANDÉN N E. and M. HENNING, Adrenergic nerve function, noradrenaline level and noradrenaline uptake in cat nictitating membrane after reserpine treatment. *Acta physiol. scand.* 1966 *67* 498—504

ANDÉN N E T MAGNUSSON and B. WALDECK, Correlation between noradrenaline uptake and adrenergic nerve function after reserpine treatment. *Life Sci.* 1964 *3* 19—25

BERTLER, Å., A. CARLSSON and E. ROSENGREN Release by reserpine of catechol amines from rabbits' hearts. *Naturwissenschaften* 1956 *22* 521

BERTLER Å., A. CARLSSON and E. ROSENGREN A method for the fluorimetric determination of adrenaline and noradrenaline in tissues. *Acta physiol. scand.* 1958 *44* 273—292.

BERTLER, Å A CARLSSON M. LINDQVIST and T MAGNUSSON On the catechol amine levels in blood plasma after stimulation of the sympathoadrenal system. *Experientia* (Basel) 1958 *14* 184

BIRGARDE, A M., A. CARPI and O A. ORRSVOLDER, Urinary excretion of catechol amines in the rat after their liberation by reserpine or dexamphetamine. *Brit J Pharmacol.* 1964. *23* 529—539

CARLSSON, A. Drugs which block the storage of 5-hydroxytryptamine and related amines I Handb d. exp. Pharmak. Erg Werk Bd. XIX. Ed. V Erspamer Springer Verlag, Berlin-Heidelberg-New York 1965 *19* 529—592.

CARLSSON A., E. BOJE RASMUSSEN and P KRISTJANSEN The urinary excretion of adrenaline and noradrenaline by schizophrenic patients during reserpine treatment *J Neurochem.* 1959 *4* 318—320

CARLSSON A N Å HILLARP and B. WALDECK, Analysis of the Mg^{++}-ATP dependent storage mechanism in the amine granules of the adrenal medulla. *Acta physiol. scand.* 1963. *59* Suppl. 215 1—38.

CARLSSON A E ROSENGREN Å. BERTLER and J NILSSON, Effect of reserpine on the metabolism of catechol amines I Psychotropic drugs Ed S Garattini and V Ghetti. Elsevier Publ. Comp Amsterdam 1957 363—372.

CARLSSON, A. and B WALDECK, The accumulation of [^{3}H]noradrenaline in the adrenergic nerve fibres of reserpine-treated mice. *J Pharm Pharmacol.* 1967 *19* 182—190.

ELMQVIST D W W HOFMANN J KUGELBERG and D. M J QUASTEL, An electrophysiological investigation of neuromuscular transmission in myasthenia gravis. *J Physiol* (Lond.) 1964 *174* 417—434

EULER, U S Noradrenaline Charles C Thomas, Springfield, Ill. 1956. pp 382.

FARRIS, E J and J Q GRIFFITH The rat in laboratory investigation. Hafner Publishing Company New York 1949 2nd ed pp 542

GADDUM J H W A KELLOG and G LAVERTY The action of reserpine on the excretion of adrenaline and noradrenaline *J Neurochem* 1958 *2* 49—255

HERTTING G J AXELROD and J G. WHITBY Effect of drugs on the uptake and metabolism of H^3-norepinephrine *J Pharmac* 1961 *134* 146—153

HILLARP N Å and T MALMFORS Reserpine and cocaine blocking of the uptake and storage mechanisms in adrenergic nerves *Life Sci.* 1964 *3* 703—708

IVERSEN L L J GLOWINSKI and J AXELROD, The uptake and storage of H^3-norepinephrine in the reserpine-pretreated rat heart *J Pharmacol.* 1965 *150* 173—183

JOHNSON G E., The effect of cold exposure on the catecholamine excretion of adrenalectomized rats treated with reserpine *Acta physiol scand.* 1963 *59* 438—444

KUFFLER, S W Electric potential changes at an isolated nerve-muscle junction *J Neurophysiol* 1942 *5* 18—26

LEDUC, J Catecholamine production and release in exposure and acclimation to cold. *Acta physiol. scand.* 1961 *53* Suppl 183 1—101

LINDMAR, R. and E. MUSCHOLL, Die Wirkung von Pharmaka auf die Elimination von Noradrenalin aus der Perfusionsflüssigkeit und die Noradrenalinaufnahme in das isolierte Herz *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak* 1964 *247* 469—492.

MUSCHOLL, E Die Hemmung der Noradrenalin-Aufnahme des Herzens durch Reserpin und die Wirkung von Tyramin *Naunyn-Schmiedeberg's Arch. exp Path. Pharmak.* 1960 *240* 234—241

MUSCHOLL, E. and M VOGT The action of reserpine on the peripheral sympathetic system *J Physiol.* (Lond.) 1958 *141* 132—155

STJÄRNE, L., Studies of catecholamine uptake storage and release mechanisms *Acta physiol. scand* 1964 *62* Suppl 228 1—97

TRENDELENBURG, U Supersensitivity and subsensitivity to sympathomimetic amines *Pharmacol. Rev* 1963 *15* 225—276.

Acta physiol. scand. 1968. 72. 139—147

From the Department of Histology University of Lund, and the Research Laboratories, AB Draco, Lund, Sweden

Function of Cholinergic Nerve Fibres in the Cat Iris Dilator

By

B. Ehinger, B. Falck and H. Persson

Received 25 May 1967

Abstract

Ehinger, B., B. Falck and H. Persson. *Function of cholinergic nerve fibres in the cat iris dilator.* Acta physiol. scand. 1968. 72. 139—147

The effect of electrical stimulation, noradrenaline, acetylcholine, isoprenaline, tyramine phenoxybenzamine dibenamine, atropine, and INPEA was investigated in normal, sympathetically and parasympathetically denervated dilator muscles. High-frequency stimulation caused the muscle to contract and low-frequency stimulation caused it to relax. The response to high-frequency stimulation was blocked by phenoxybenzamine, dibenamine and sympathectomy while the response to low frequency stimulation was blocked by atropine and parasympathectomy. Acetylcholine gave an initial relaxation followed by a slow contraction. The latter was blocked by phenoxybenzamine. After sympathetic denervation, acetylcholine in moderate doses elicited relaxations only. Atropine in moderate concentrations depressed the response to noradrenaline and to high-frequency stimulation. At lower doses, the effect was reversed: atropine potentiated the response to noradrenaline and high-frequency stimulation. This potentiation was abolished by parasympathectomy. It is concluded that the dilator pupillae is innervated by both adrenergic and cholinergic fibres, and an interaction between the two fibre types is not excluded.

The classical concept of the innervation of the iris is that the sphincter is supplied by parasympathetic, cholinergic fibres, while the dilator is supplied by sympathetic, adrenergic fibres. It was discussed whether or not the sphincter also contained sympathetic fibres, but until more advanced histochemical and pharmacological methods of study became available the weight of evidence pointed to a simple innervation (Loewenfeld 1958). There is now irrevocable evidence (Laties and Jacobowitz 1964, Ehinger 1964a and b, Schaeppi and Koella 1964a, Laties and Jacobowitz 1966, Ehinger 1966a (for further references see Ehinger 1966b)) that sympathetic, adrenergic fibres do reach the sphincter muscle in all species studied, humans included.

While the supply of adrenergic fibres in the sphincter thus had been the subject of some controversy before being established, it has always been next to universally accepted that the dilator is supplied by only sympathetic adrenergic fibres. How-

ever recent studies with a variety of techniques, including selective denervation, have unequivocally established that cholinergic, parasympathetic fibres occur in and at the dilator of mice (Ehinger and Sporrong 1966) rats (Ehinger and Falck 1965 and 1966 Eränkö and Räisänen 1965 Eränkö 1966 Ehinger Sporrong and Stenevi 1967) and cats (Laties and Jacobowitz 1966, Ehinger 1967) and also made their presence highly probable in a number of other species (Laties and Jacobowitz 1964, Lukáš 1964 Ehinger 1966c) primates included (Laties and Jacobowitz 1966) It has further been demonstrated that the adrenergic and cholinergic fibres run tightly intertwined in a hitherto unknown manner (Ehinger and Falck 1965 and 1966, Ehinger et al. 1967) The function of these cholinergic fibres of the dilator and the significance of the close relationship between the adrenergic and cholinergic fibres was, however not adequately known and this deficiency initiated the present work

Materials and methods

34 randomly selected cats were used. 9 were subjected to unilateral parasympathectomy by excision of the ciliary ganglion (Shen and Cannon 1936) and 7 to unilateral cervical sympathectomy in both cases 2—6 weeks before killing the animal. The extirpation of the ciliary ganglion has been shown not to influence the sympathetic innervation detectably (Ehinger 1967) Two radial strips were cut from the iris, one from the temporal and one from the nasal side i order t avoid as much as possible interference from the sphincter which extends all the way t the iris root in the upper and lower iris quadrants (Walls 1942) A thin cotton thread was tied around the iris strip 1—1.5 mm from the pupillary margin. Another thread was tied around the iris root t the base of the pectinate ligament.

The iris strip was suspended in a bath with Krebs-Ringer solution containing glucose, 1 mg/ml. The solution was well aerated with gas mixture containing 95 % oxygen and 5 % carbon dioxide Denervated muscles were always run in the same bath as normal control muscle from the same animal. Contractions of the muscles were recorded on a Grass polygraph model 7 with FTO3C transducers. One end of the muscle was fastened t platinum hook serving as electrode The other electrode was placed in sliding contact with the other end of the muscle. The stimulator was Grass S 5 and the stimulation pulses were monitored on a Tektronix 564 oscilloscope. The applied voltage varied between 1 and 3 olts in the routine . Care was taken to ensure that the contractions elicited in the muscles were always well bmaximal. The stimulus strength was individually adapted for each muscle. Owing to this and the varying strengths of the muscle strips, the absolute forces varied for the statistical treatment the esults had then t be expressed as per cent changes the contraction forces of the individual muscles. The contraction forces given in the table are the mean of tw or more tests on each muscle setup. Resting muscle tensions varied between 70 and 240 mg The drugs used were noradrenaline bitartrate atropine sulphate INPEA [1 (4-nitrophenyl)-2-isopropylaminoethanol], phenoxybenzamine HCl (Smith, Kline and French through Mekos AB, Hälsingborg) dibenamine HCl (Smith, Kline and French through Mekos AB, Hälsingborg), isoprenaline sulphat and acetylcholine chloride. The concentrations are given as the mentioned salts, with the exception of noradrenaline which is given as the base. The preparations were always rested more than 10 min between each injection. Atropine was alway left in the bath more than 20 min before its effect was tested.

Sections stained for cholinesterases ccording to the Holmstedt modification of the Koelle technique (Holmstedt 1957) readily demonstrat the arious muscles of the iris, since they contain high amounts of this enzyme. Three of the preparations actually used were serially sectioned and stained for acetylcholinesterase In no case did the working part of the preparations show signs of any sphincter or ciliary muscle fibres.

Results

Normal muscles

Noradrenaline in concentrations 1—8 µg/ml caused contractions with forces ranging about 20 to 80 mg (Fig 1) The onset was rapid and the contractions usually

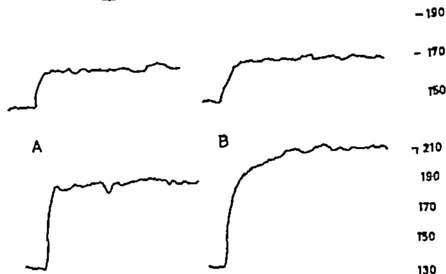


Fig. 1 Cat iris dilator Below normal control muscle above, parasympathetically denervated. Effect of noradrenaline (6 µg/ml) A, before and B, after atropine (0.06 µg/ml) Calibration in mg.

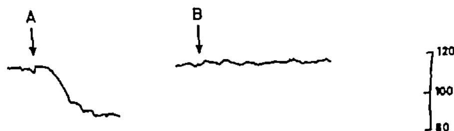


Fig 2. Cat iris dilator Effect of isoprenaline (3 µg/ml) A before and B after INPEA (20 µg/ml) Calibration in mg.

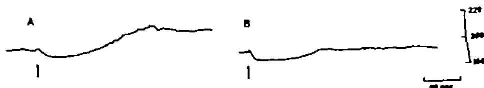


Fig. 3. Cat iris dilator Effect of acetylcholine (10 µg/ml) A before and B after treatment with phenoxybenzamine (1 µg/ml) Calibration in mg

reached a steady level, which increased with increasing noradrenaline dose and which was maintained until the wash. The contractions were ffecti ely blocked by phenoxybenzamine or dibenamine, 1 µg/ml.

Isoprenaline (3 µg ml) regularly caused relaxations, 10—30 mg (Fig) The effect was readily and effectively blocked by INPEA (20 µg/ml)

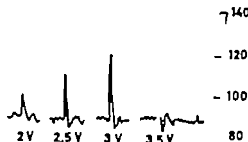


Fig. 4 Cat iris dilator Response to high-frequency stimulation at various voltages. Calibration in mg. Stimulation time 2 sec.

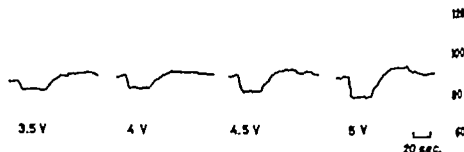


Fig. 5. Cat iris dilator Response to low-frequency stimulation at various voltages. Calibration in mg

Tyramine (10—100 µg/ml) regularly caused contractions ranging in forces between 20 and 80 mg

Acetylcholine (10—100 µg/ml) usually caused a slight relaxation (10—15 mg) followed by a slow contraction. The contraction could be blocked by phenoxybenzamine 1 µg/ml (Fig 3)

Electrical stimulation gave different responses with different frequencies. In the region 8—100 cps (high frequency stimulation) the muscle contracted rapidly The contraction force varied with the voltage applied over the electrodes (Fig 4). After dibenamine (1 µg/ml) phenoxybenzamine (1 µg/ml) or sympathectomy the contraction disappeared and was replaced by a relaxation. Low frequency (0.5—4 cps) stimulation caused a relaxation which also varied with the applied voltage (Fig 5) Often small contractions synchronous with the stimulation frequency were observed superimposed on the relaxation, as noted also by Schaeppi and Koella (1964b) The occurrence of the relaxations was very sensitive to the degree of distension of the muscle and could, at times, be very difficult to elicit. Atropine or parasympathectomy blocked the response, and usually converted it into a slight contraction or a series of minimal contractions synchronous with the stimulation (Fig 6) Hexamethonium (10 µg/ml) did not affect the relaxations measurably Phenoxybenzamine (1 µg/ml) appeared to enhance the response slightly while INPEA was without effect on it.

Atropine in concentrations down to 0 1 µg/ml caused a decrease in the response to both high frequency stimulation and noradrenaline. This agrees with previous ob-



Fig. 6. Cat iris dilator. Low-frequency stimulation. A before and B after atropine 0.06 µg/ml. Calibration in mg.

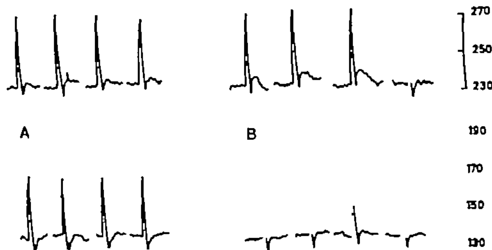


Fig. 7. Cat iris dilator. Below normal control muscle, above parasympathetically denervated. Effect of high-frequency stimulation. A before and B after atropine (0.06 µg/ml). Stimulation time 2 sec. Calibration in mg.

TABLE I. Effect of atropine (0.06 µg/ml) on the response to stimulation with noradrenaline and high frequency

	Noradrenaline stimulation		High-frequency stimulation	
	Normal	Ciliary ganglionectomy	Normal	Ciliary ganglionectomy
Number of muscles	17	6	10	8
Change in contraction force after atropine ± s.e.m.	+20.4 ± 7.6	−3.2 ± 3.9	+34.6 % ± 7.9	+3.0 ± 6.1 %
Significance of difference normal and denervated muscles	$p < 0.02$		p 0.01	

dilator are reached by a dual set of fibres sympathetic adrenergic ones, which stimulate it to contract and parasympathetic, cholinergic ones, relaxing the dilator.

The influence of atropine on the response to sympathomimetics and sympathetic stimulation has previously received attention both in the iris (Schaeppi and Koella 1964b) and other effector cells (see Luduena and Branin 1966) Its effect at high dose levels is usually inhibiting or adrenolytic. This was found also in the present study with concentrations above 0 1 µg/ml However at lower concentrations (0.01–0 06 µg/ml) the effect of atropine was opposite to that at higher doses, giving potentiation both in the response to noradrenaline and to electrical stimulation. It seems likely that this effect is caused by the specific cholinergic blocking activity of atropine while at higher concentrations a non-specific inhibitory effect masks the potentiation. This view is supported by the observation that after selective parasympathectomy no augmentation of the response could be obtained by the low dose of atropine, while the depression of the response with higher doses of atropine was still seen.

The potentiation by atropine of the response to high-frequency stimulation is readily understood on the basis that both adrenergic fibres (agonists) and cholinergic fibres (antagonists) reach the muscle as proposed above. The high-frequency stimulation most presumably elicits signals in both fibre types. The contraction is enhanced when the effect of the antagonists is blocked by atropine. This enhancement is of course not to be expected when the cholinergic fibres have been removed by selective parasympathectomy which proved to be the case. The cause of the potentiation by atropine on the response to noradrenaline is less readily seen. It is clear that intact cholinergic fibres are necessary for the response, since it is abolished by selective parasympathectomy Cholinergic fibres are usually believed to release acethylcholine spontaneously (see Emmelin 1967) The effect of this continuous inhibitory activity would be blocked by atropine, resulting in an enhanced response to noradrenaline It is however not known whether such a subliminal release would be sufficient to explain the increase in contraction force, which is around 30 %. Another possibility arises from a recent hypothesis on the function of peripheral vegetative nerves (Ehinger and Falck 1967) Adrenergic and cholinergic fibres were postulated to be able to influence each other through some kind of axo-axonal contact, implying that there are adrenergic receptors on the cholinergic fibre. When added to the bath noradrenaline would stimulate both the muscle and the inhibitory cholinergic fibres. Atropine blocks the effect of the inhibitory fibres, so that the response to noradrenaline is enhanced.

This study has been supported by grants from the United States Public Health Service (grant NB 06701-01) by the Swedish Medical Research Council (project B67 12X 712-02A) and by the Faculty of Medicine University of Lund, Sweden.

References

ALBERTANTE, L. and W. M. [illegible], Relationship between configuration and adrenergic β-receptor blocking activity of optical isomers of 1-(4-nitrophenyl) 2-isopropylaminoetanol (INPEA). *J med Chem.* 1966. 9 650—653

ALPHEN, G. W. H. M. VAN, S. L. ROBINETTE and F. J. MACRI, The adrenergic receptors of the intraocular muscles of the cat. *Int. J. Neuropharmacol.* 1964 *2* 259—272

ALPHEN, G. W. H. M. VAN, R. KERN and S. L. ROBINETTE, Adrenergic receptors of the intraocular muscles. *Arch. Ophthal.* (Chicago) 1965. *74* 253—259

BURN, J. H., J. J. DROMEY and B. J. LARGE, The release of acetylcholine by sympathetic nerve stimulation at different frequencies. *Brit. J. Pharmacol.* 1963 *21* 97—103

EHINGER, B., Distribution of adrenergic nerves to orbital structures. *Acta physiol. scand.* 1964a. *62* 291—292.

EHINGER, B. Adrenergic nerves to the eye and its adnexa in rabbit and guinea-pig. *Acta Univ. Lund.* Sectio II. 1964b No. 20. 1—23

EHINGER, B. Adrenergic nerves to the eye and to related structures in man and in the cynomolgus monkey. *Invest. Ophthal.* 1966a. *5* 42—52.

EHINGER, B. Ocular and orbital vegetative nerves. *Acta physiol. scand.* 1966b. *67* Suppl. 268. 1—35

EHINGER, B. Cholinesterases in ocular and orbital tissues of some mammals. *Acta Univ. Lund.* Sectio II 1966c. No. 2. 1—15

EHINGER, B., Double innervation of the feline iris dilator. *Arch. Ophthal.* (Chicago) 1967 *77* 541—545.

EHINGER, B. and B. FALCK, Noradrenaline and cholinesterases in concomitant nerve fibres in the rat iris. *Life Sci.* 1965 *4* 2097—2100

EHINGER, B. and B. FALCK, Concomitant adrenergic and parasympathetic fibres in the rat iris. *Acta physiol. scand.* 1966. *67* 201—207

EHINGER, B. and B. FALCK, Interactions between adrenergic and cholinergic peripheral nerve fibres. 1967 To be published.

EHINGER, B and B SPORRONG, Adrenergic and cholinergic axons in the mouse iris. *Experientia* (Basel) 1966 *22* 218.

EHINGER, B., B SPORRONG and U STENEVI, Combining the catecholamine fluorescence and methylene blue staining methods of demonstrating nerve fibres. *Life Sci* 1967 *6* 1973—1974

EMMELIN, N., Nervous control of the salivary glands. In *Handbook of Physiology Alimentary Canal I* 1967 Williams and Wilkins Baltimore. In press.

ERÄNKÖ, O Demonstration of catecholamines and cholinesterase in the same section. *Pharmacol.* 1966 *18* 353—358.

ERÄNKÖ, O and L. RÄISÄNEN, Fibres containing both noradrenaline and acetylcholinesterase in the nerve net of the rat iris. *Acta physiol. scand.* 1965 *63* 505—506.

FERRY, C. B Cholinergic link hypothesis in adrenergic neuroeffector transmission. *Physiol. Rev.* 1966 *46* 420—456

GARRY, R. C. and J. S. GILLESPIE, The responses of the musculature of the colon of the rabbit to stimulation, *in vitro* of the parasympathetic and of the sympathetic outflows. *J. Physiol.* (Lond.) 1955 *128* 557—576.

HOLMSTEDT, B A modification of the thiocholine method for the determination of cholinesterase II. Histochemical application. *Acta physiol. scand.* 1957 *40* 331—337

LATIES, A. and D. JACOBOWITZ, A histochemical study of the adrenergic and cholinergic innervation of the anterior segment of the rabbit eye. *Invest. Ophthal.* 1964 *3* 592—600.

LATIES, A. and D. JACOBOWITZ, A comparative study of the autonomic innervation of the eye in monkey, cat and rabbit. *Anat. Rec.* 1966. *156* 383—396

LOWENFELD, I. Mechanisms of reflex dilatation of the pupil. *Docum. ophthal.* (Den Haag) 1958. *XII* 185—448

LUDUENA, F. P. and M. J. BRANIN, Adrenolytic activity of atropine, (+)-hyoscyamine, atropine homatropine, and related compounds. *J. Pharmacent. Sci.* 1966. *55* 280—284

LUKÁŠ, Z. Die Cholinesteraseaktivität und Innervation der inneren Augenmuskeln. *Z. mikr.-anat. Forsch.* 1964 *71* 331—338.

RUSKELL, J. C. Untersuchungen am normalen und am chronisch denervierten isolierten Iris-Dilatator mit der Methode der Luftströmung. *Helv. physiol. acta* 1955 *13* 309—318

SCHAEPPI, U. and W. P. KOELLA, Adrenergic innervation of cat iris sphincter. *Amer. J. Physiol.* 1964a. *207* 273—279

SCHAEPPI, U. and W. P. KOELLA, Innervation of the cat iris dilator. *Amer. J. Physiol.* 1964b *207* 1411—1416

SIMEONE, F. C. and W. B. CANNON, Sensitization of the denervated pupillary sphincter to acetylcholine. *Chin. J. Physiol.* 1936. *10* 359—372.

WALLS, G. *The vertebrate eye and its adaptive radiation.* 1942 Cranbrook Institute of Science Bloomfield Hills, Michigan.

Acta Physiol. scand. 1968. 72. 148—156
F om the Department of Physiology University of Göteborg, Sweden

Mechanism of Escape of Skeletal Muscle Resistance Vessels from the Influence of Sympathetic Cholinergic Vasodilator Fibre Activity

By
A. M. Djojosugito[1] B. Folkow B. Lisander and H. Sparks[2]

Received 30 May 1967

Abstract

Djojosugito A. B. Folkow B. Lisander and H. Sparks. *Mechanism of escape of skeletal muscle resistance vessels from the influence of sympathetic cholinergic vasodilator fibre activity.* Acta physiol. scand. 1968. 72. 148—156.

Experiments on the all muscle irculation of cats and dogs indicate that the return of muscle blood flow to control lev l during sympathetic asodilator fibre stimulation (the escape phenomenon) occurs only when autoregulation of flow n response to pressure changes is well dev loped, when ascular smooth muscle ctivity is pronounced. When this is not the case sustained vasodilatation is induced by prolonged activation of these fibres. The escape is therefore dependent on ou terregulation of the ascular effectors themselves. Precapillary sphincter acti ty is increased by but does not significantly affect, the neurogenically reduced flow esistanc F rther sphincter activity returns to control when the escape develops, showing that this phenomenon is property of smooth muscle located upstream of he sph ncters in the prec pillary resistance essels prope The ombined evidence suggests ely estricted t of action of the sympathetic hol nergic asodilator fibres, directly suppressing only pacemaker smooth muscle cells and/or key sites of the petreams myogenic propaga ion of exc ation from such pacemakers S nce pacemake activity seems to be best dev loped in the smallest precapillary resistance essels, neurogenic inhibitory action at this site would lead to dilatation of proximally situated resistance sections, which for their basal tone ar depende on propagated excitation Escape would then occur if latent pacemakers in this resistanc sec ion graduall became dominant and reestablished myogenic tone. This mechanism n sim la to the gus escape phenomenon of the heart.

The increased blood flow associated with sympathetic vasodilator fibre activation usually lasts one or two minutes at most and then, in spite of continued vasodilator fibre stimulation, uscle blood flow escapes to control values (e.g. Folkow lander and Oberg 1961 This occurs irrespective of the method used f blockade of the adrene gic sympathetic fibres and cannot be ascri to insuf α-blockade Neither is this vasodilator fibre escape due to ta l laxis of

[1] Department of Physiology Faculty of Medicine, Gadjah Mada Universi
On fellowship from the W H O
[2] Department of Physiology The University of Michigan, East Medical B
48104 USA.

lar smooth muscle to acetylcholine since prolonged infusion of acetylcholine results in continued vasodilatation. Further the escape can hardly be ascribed to depletion of the transmitter at the nerve endings because after eserine, acetylcholine can be detected in the blood stream in considerable amounts for stimulation periods longer than one or two minutes (Folkow, Haeger and Uvnäs 1948). Lastly local damage to nerves seems to be ruled out, first by the fact that hypothalamic activation of the vasodilator fibres also results in increased blood flow followed by escape and, second, by the fact that well-sustained vasoconstrictor fibre effects are induced by the same stimulation characteristics if no α-blocking drugs are given.

The hypothesis that the escape from vasodilator fibre stimulation is due to a local vascular autoregulatory mechanism has been tested and supported by the experiments reported below.

Methods

The experiments were performed on the circulation of calf muscles (Kjellmer 1964) of 16 cats and 4 dogs, anesthetized with chloralose (60 mg/kg), chloralose and urethane (50 and 100 mg/kg) or nembutal (30—40 mg/kg).

The animal was heparinized[1] and the entire venous outflow of the calf muscle preparation was recorded continuously by passing the blood from the popliteal vein through a drop recorder. Blood was returned to the animal via a funnel in the femoral vein. This open system allowed adjustments of the venous outflow pressure so that capillary filtration coefficient (CFC) could be measured (see below). Arterial inflow pressure was regulated by a clamp around the abdominal aorta and measured through a cannula in the contralateral femoral artery. Intra-arterial injections and infusions were given via a catheter inserted into the central end of the inferior mesenteric artery or via a small side branch of the femoral artery supplying the calf muscle preparation. Venous outflow pressure was recorded by means of a side tube connected to the venous outflow apparatus.

For determination of CFC the calf was placed in a watertight plethysmograph permitting continuous recording of tissue volume (Mellander 1960). CFC was determined by raising the venous outflow pressure a known amount and noting the rate of outward filtration resulting from the increased capillary hydrostatic pressure (see Pappenheimer and Soto Rivera 1948, Mellander 1960). CFC reflects both the capillary surface area and capillary permeability. However, since there is nothing to indicate that acetylcholine affects capillary permeability (e.g. Kjellmer 1965) it was concluded that the usually transient changes in CFC upon vasodilator fibre stimulation in all probability reflect changes in capillary surface area alone. Capillary surface area is determined by the number of open capillaries which, in turn, is controlled by the net tone of the precapillary sphincters.

The abdomen was opened in the midline, the intestine extirpated and the sympathetic chain isolated and transected in the high lumbar area. A bipolar silver electrode was placed around the chain at the level of L4—L5. To ensure complete blockade of the adrenergic vasoconstrictor fibres supramaximal doses of guanethidine[1], phenoxybenzamine[1] and phentolamine were given in combination, usually intra-arterially to the calf muscle preparation, to ensure a very high concentration of these adrenergic blocking drugs in this vascular bed. Subsequent stimulation of the sympathetic chain results in a cholinergic vasodilatation which is entirely blocked by atropine. In order to be sure that the vasoconstrictor fibres were completely blocked, tyramine was given at the end of each experiment and preparations which exhibited residual vasoconstrictor activity were rejected.

Results

Autoregulation of flow and the escape. A kymograph record demonstrating the typical escape pattern, which usually occurs during stimulation of the cholinergic vasodilator fibres after complete constrictor fibre block, is shown in Part I of Fig. 1.

[1] We are indebted to Vitrum, Stockholm, for generous supply of heparin.
[1] We are indebted to Ciba, Stockholm, and to Smith, Kline & French, USA, for generous supply of these drugs.

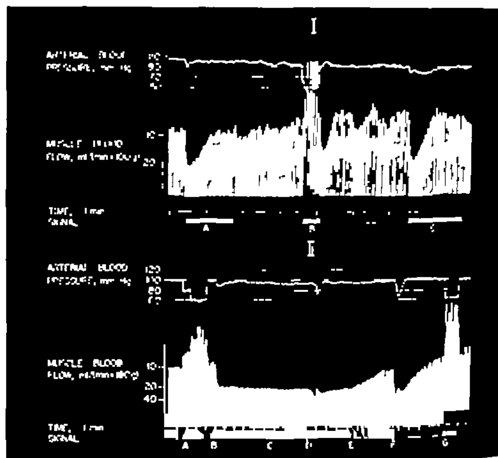


Fig. 1. Effects of sympathetic dilator fibre stimulation on muscle resistance vessels in different states of reactivity.

I. Cat 2.7 kg. Regional constrictor fibres completely blocked by intra-arterial infusion of 5 mg guanethidine per kg body weight. *A* and *C* show the effects of dilator fibre stimulation (8 imp/sec, 2 msec, 7 V); note complete escape from the dilator fibre influence within 40 seconds and the absence of any overshoot in vascular tone on interruption of the stimulation. *B* illustrates the considerable reactivity of the resistance vessels in form of a pronounced flow autoregulation to pressure changes.

II. Cat 4.6 kg. Regional constrictor fibres completely blocked by intra-arterial infusion of 5 mg guanethidine and 1 mg phentolamine per kg body weight. *A* and *G* illustrate poor vascular "reactivity" by means of the virtual absence of any autoregulation of flow to pressure changes. At *B* vasodilator fibre stimulation is started at 8 imp/sec, 2 msec, 7 V, frequency being increased to 10 imp/sec at *C* with interruption of the stimulation at *E*. Note sustained vasodilatation for the fourteen minute period of stimulation and the slow regain of vascular tone on interruption of the stimulation. At *D* and *F* acetylcholine 0.1 µg is injected intra-arterially.

Resting blood flow of this denervated skeletal muscle bed was approximately 9 ml/min × 100 g with an arterial pressure of 100 mm Hg. The lumbar sympathetic chain was stimulated (8 imp/sec, 2 msec duration, volts) at the time indicated by the deflection of the event signal at A. Flow increased within a few seconds to more than 20 ml/min × 100 g, but, in spite of continued stimulation, returned to the control level within one minute.

In order to assess the ability of the vascular bed to autoregulate flow in the face

of altered perfusion pressure, i.e. to evaluate vascular smooth muscle reactivity arterial pressure was reduced at B. The decrease in arterial pressure to 40—50 mm Hg caused an immediate decrease in flow but during the period of decreased pressure, the flow increased towards normal. When the perfusion pressure was suddenly returned to normal there was a transient increase in blood flow which reveals the extent of the compensatory dilation of the resistance vessels during the period of reduced pressure and flow In other words, the vascular bed exhibits considerable autoregulation of flow and the reactivity of the precapillary smooth muscles is therefore considerable.

At C the sympathetic stimulation was repeated, and again blood flow escaped from the continued vasodilator fibre activation and returned to the control value A characteristic feature is the lack of any secondary overshoot in vascular tone when the dilator fibre stimulation is interrupted. In this respect the response pattern simulates that produced when true tachyphylaxis to a vasoacti e agent is in ol ed. However the prolonged vasodilatation during acetylcholine infusion shows that no tachyphylaxis to the cholinergic transmitter occurs. Occasionally a moderate degree of autoregulation to infused acetylcholine is seen, but then there is always a fairly pronounced overshoot in ascular tone upon interruption of the infusion.

Part II of Fig 1 is a kymograph record taken from an experiment in which the skeletal muscle vascular bed did not exhibit autoregulation of flow i.e. vascular smooth muscle reactivity is quite low Arterial pressure was reduced in 20 mm Hg steps to 60 mm Hg and there was no tendency for flow to increase during the periods of reduced pressure. In addition, when the arterial pressure was returned to 100 mm Hg no reactive hyperemia was present. In this case guanethidine and phentolamine had been given as an intra-arterial infusion to the calf preparation, which had interfered with an earlier vivid blood flow autoregulation at the same time that the adrenergic vasoconstrictor fibres were blocked. This interference with vascular reactivity" is quite probably nonspecific and unrelated *per se* to the α-blocking effect, since in other experiments complete α-blocking effect was obtained by other combinations of α-blockers with no interference with vascula reactivity to pressure changes. It is possible that the depressing effect on vascular smooth muscle reactivity is related to the quinidine-like effect that phentolamine ppears to exert. Whatever the case this effect proved of ad antage for the further analysis of the vasodilator fibre influence.

At B in part II Fig 1 the lumbar chain was stimulated (8 imp sec msec duration, 7 olts, later increased to 10 imp sec) and blood flow increased from 10 ml min × 100 g reaching a alue slightly bov 20 ml min 100 g This sympathetic stimulation continued for 14 min and during the whole period blood flow remained elevated. At E, when the stimulation was discontinued, blood flow returned sluggishly to the control value At D and F acetylcholine 0.1 μg was injected intra-arterially to compar its effect w th that of the dilator fibres. The passive response of the resistance vessels to the reduction f the rterial pressure at G again indicates that this bed cannot autoregulat flow when perfusion pressure

is lowered. However later on in the experiment the vessels again began to autoregulate following pressure changes and along with this the vasodilator fibre effect became more and more transient.

It was observed repeatedly that lack of autoregulation of blood flow in response to altered perfusion pressure is associated with absence of the blood flow escape during sympathetic vasodilator stimulation. At this time very sustained vasodilatations are obtained by activation of these fibres and there are certainly no signs of any tachyphylaxis to the cholinergic transmitter or to any "exhaustion" of the dilator fibres.

Precapillary sphincter tone during vasodilator fibre stimulation. Since vasodilator fibre escape is associated with the presence of intact autoregulation of flow one possible mechanism for the escape might be a compensatory increase in precapillary sphincter tone induced by the increased transmural pressure downstream after resistance vessel dilation and/or to the consequent washout of vasodilator metabolites by the increased blood flow. If pronounced enough such a secondary downstream constriction might increase *total flow resistance* enough to mask the upstream neurogenic dilatation.

Fig. 2 graphically summarizes the results of these experiments and compares the CFCs determined during periods of increased flow due to vasodilator fibre activity with those during periods of increased flow during the infusion of acetylcholine. Acetylcholine infusion results in increased CFC along with the increased blood flow (see also Kjellmer 1965 and Mellander 1966). On the other hand the increased

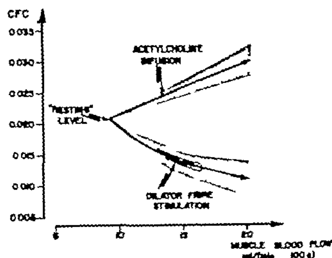


Fig. 2. Diagrammatic illustration of the effects of acetylcholine infusion and of sympathetic vasodilator fibre stimulation on muscle blood flow and CFC. The "resting" level gives the mean value of 36 measurements. The directions of the arrows indicate the general correlation between flow increase and CFC changes as related to the "resting" level, the hatched areas giving the limits of the double S.D. for the values obtained. Note how CFC regularly increases in good proportion to the flow increase when acetylcholine is infused, while CFC as regularly decreases when the vasodilator fibres are stimulated. The thin looped arrow is intended to illustrate that, on escape of the resistance vessels from the dilator fibre influence CFC returns towards control instead of being further decreased.

blood flow caused by vasodilator fibre stimulation is indeed regularly associated with a *decreased* CFC. It is important to stress however that the more the neurogenic blood flow increase escapes and returns towards normal during continued vasodilator fibre stimulation, the more CFC returns to control level, and in close parallel. Thus, the largest decrease in CFC was seen when considerable, fairly sustained flow increases were obtained by dilator fibre stimulation, occurring in experiments where the resistance vessels exhibited little or no autoregulation to pressure changes.

These results indicate that the increased precapillary sphincter tone found along with the neurogenically increased blood flow is not responsible for the vasodilator escape. If it were, CFC would, if anything, show a further decrease when the escape from the vasodilator fibre stimulation develops and would not be present just during the period of increased flow. However when the escape developed, CFC always returned towards normal the regain of vascular tone, responsible of the escape phenomenon, must therefore take place elsewhere. It definitely occurs on the precapillary side since there was never any sign of increased capillary pressure as a consequence of the escape. Had the escape been due to a postcapillary compensatory constriction there would have been a considerable rise in capillary pressure.

Discussion

The results of the present study indicate that the vasodilator fibre escape accompanies closely the ability of the vascular bed to autoregulate i.e. a considerable vascular smooth muscle reactivity appears to be a prerequisite for the escape phenomenon to be established. One possible mechanism is increased precapillary sphincter tone offering increased resistance to flow since the present measurements of CFC and other indicators of capillary exchange (Rosell and Uvnäs 1962, Renkin and Rosell 1962) suggest decreased capillary surface area with the onset of increased flow. Such a mechanism cannot, however be responsible of the escape because when this escape causes flow to return to control level during stimulation, CFC returns to control levels also. Had the adjustments of the precapillary sphincter sections been responsible the CFC decrease would have become further accentuated with the escape. It appears then, that the increased precapillary sphincter tone results from the resistance vessel dilation and lasts no longer than the increased flow. Thus, the largest decrease in CFC was seen when there was no perceptible escape from the dilator fibre. The possible occurrence of a secondary postcapillary constriction is ruled out by the fact that there was no sign of any increase in capillary pressure level in connection with the escape.

What, then, is the explanation for the escape from vasodilator fibre activity? The tendency for the vascular smooth muscles of the resistance vessels to autoregulate flow in response to alterations in perfusion pressure requires a considerable reactivity of these muscles and seems to be intimately connected with their ability to exhibit myogenic tone (Folkow 1953, 1964). One explanation for the myogenic tone within the precapillary resistance section is that smooth muscle cells predominan

in the smallest vessels close to the capillary section possess inherent pacemaker activity as do gut and ureteral smooth muscle and that normally this unsynchronized pacemaker activity by means of cell-to-cell propagation, is spread in the proximal direction wherever the smooth muscle cover is continuous. This unsynchronized activity propagated from peripheral branches in the proximal direction, would then tend to become fused when vascular arborizations are reached hence creating a fairly steady level of myogenic tone in somewhat larger vessels (Folkow 1964). Pacemaker activity and cell to-cell propagation of myogenic activity has been directly shown to occur in isolated strips from small arteries (Johansson and Bohr 1966) Decreasing autoregulatory activity could be accomplished, then, by decreasing spontaneous pacemaker activity of vascular smooth muscle ("negative chronotropic influence") and or by depressing the ability to exhibit cell-to-cell propagation ("negative dromotropic influence")

Pacemaker activity in gut and ureter normally dominate in certain areas and only when this pacemaker activity becomes locally suppressed do other areas assume this role by means of earlier "latent pacemakers. It would be reasonable to assume that a similar pattern exists in the small resistance vessels such events are certainly seen in the myocardium. Neurogenic inhibition of myogenic activity in the precapillary resistance vessels could, in fact, well be accomplished by a very restricted spatial distribution of cholinergic fibre endings, if these endings are concentrated in or closely proximal to the areas of high pacemaker activity In general, such pacemaker activity seems to be best developed in the smallest precapillary resistance vessels, close to the capillary bed Such an arrangement of the cholinergic vasodilator fibres could account for the difficulty in locating them in random sections of skeletal muscle blood vessel Further if a widespread distribution along the resistance vessels were present, it would be difficult to explain the often complete escape from their influence since acetylcholine infusion to the same vascular bed certainly can produce a sustained vasodilatation

We propose then that vasodilation, due to stimulation of cholinergic sympathetic fibres, may be due to the release of acetylcholine from nerve terminals concentrated around or just proximal to, those smooth muscle cells in the small precapillary vessels which primarily function as pacemakers and normally propagate their activity in the proximal direction Their spontaneous rhythmicity ceases and or the transmission of this activity in the proximal direction becomes blocked and as a consequence the corresponding resistance sections relax and a considerable fall in resistance to flow ensues. However as long as smooth muscle reactivity is good, other potential pacemaker cells placed proximal to the site of action of the dilator fibres begin to exhibit automaticity and myogenic tone then returns in the initially relaxed vascular sections. This escape is, in principle an analogue to the well known "vagus escape" phenomenon of the heart, where ventricular pacemakers can take over and drive the ventricles after vagal fibre activation has suppressed auricular pacemakers and or blocked the transmission over the A—V node.

This concept is supported by the observation that blood flow usually does not

escape during prolonged infusions of acetylcholine when all smooth muscle cells must be affected. As mentioned above there is occasionally a tendency for escape when acetylcholine is infused, but then the interruption of the infusion always leads to a considerable overshoot in vascular tone. This is never the case in escape from the dilator fibre influence (provided that the constrictor fibres have been fully blocked, so that these fibres cannot induce any counteracting adrenergic constriction)

This overshoot after acetylcholine infusion reveals a competition between the blood-borne dilator agent and counterregulating local adjustments. The absence of any overshoot after the far more marked escape from the dilator fibre influence rather suggests that initially inhibited smooth muscle cells that are not directly affected by the dilator fibre transmitter have regained their initial level of activity and reinstituted vascular tone except in the original pacemaker area, directly exposed to the cholinergic transmitter When, on the other hand, myogenic activity and thus autoregulation, is suppressed (as in Fig 1 Part II) the escape from the dilator fibre influence is also abolished, because then the potential secondary pacemakers will not become spontaneously active. Hence, a sustained and often considerable flow increase can be maintained for long periods by dilator fibre stimulation. The sluggish return of blood flow to the resting level then seen after cessation of the stimulation, may be an indication of the depressed pacemaker activity

Clearly the postulated restricted site of direct action of the dilator fibres must be proximal to the precapillary sphincters, since this section of the precapillary vessels always show a compensatory increase in activity when the neurogenic dilatation is induced. However these sphincters become dilated if acetylcholine is diffusely administered via the blood stream. Further even supramaximal activation of the cholinergic dilator fibres seems unable to produce maximal vasodilatation in the skeletal muscles, though 70—80 per cent of this maximal dilatation can be reached (Folkow Mellander and Öberg 1961) This means that they will affect the greater part of the precapillary resistance vessels though, no doubt, the precapillary sphincters are left uninfluenced and possibly also closely adjacent resistance sections in other words, the dilator fibres affect mainly medium sized and large resistance vessels. Results with another technique lend further support to such a view (Folkow Sonnenschein and Wright 1967)

Although the evidence supporting this hypothesis is circumstantial, the system proposed appears to fit the findings best and it would constitute a close parallel to the cholinergic nerve influence on another part of the cardiovascular system, the heart. Final proof depends, to a large extent, on elucidation of pacemaker and propagation properties of the resistance vessels of skeletal muscle and, of course, on direct visualization of the cholinergic vasodilator fibres, which so far have escaped morphological demonstration. However if the present hypothesis is correct, it is not surprising that these fibres have not been seen, since a very restricted distribution of only few fibres would be needed to accomplish widespread and profound dilatation.

This study was supported by grants from the Swedish Medical Research Council (B67-14X-16-03B), from Air Force School of Aerospace Medicine under Contract AF 61(052)-772 through the European Office of Aerospace Research (OAR), United States Air Force, and by a grant from U.S. Public Health (HE-05675-06).

References

FOLKOW, B., A study of the factors influencing the tone of denervated blood vessels perfused at various pressures. *Acta physiol. scand.* 1952. *27*. 99—117.

FOLKOW, B., K. HAEGER and B. UVNÄS, Cholinergic vasodilator nerves in the sympathetic outflow to the muscles of the hind limbs of the cat. *Acta physiol. scand.* 1948. *15*. 401—411.

FOLKOW, B., S. MELLANDER and B. ÖBERG, The range of effect of the sympathetic vasodilator fibres with regard to consecutive sections of the muscle vessels. *Acta physiol. scand.* 1961. *53*. 7—22.

FOLKOW, B., R. R. SONNENSCHEIN and D. L. WRIGHT, *Differential influences of nervous and local humoral factors on large and small precapillary vessels of skeletal muscle.* A symposium on circulation in skeletal muscle. Smolenice, Czech. 1966. To be publ.

JOHANSSON, B. and D. F. BOHR, Rhythmic activity in smooth muscle from small subcutaneous arteries. *Amer. J. Physiol.* 1966. *210*. Sec. 4. 801—806.

KJELLMER, I., The effect of exercise on the vascular bed of skeletal muscle. *Acta physiol. scand.* 1964. *6*. 18—30.

KJELLMER, I. and H. ODELRAM, The effect of some physiological vasodilators on the vascular bed of skeletal muscle. *Acta physiol. scand.* 1965. *63*. 94—102.

MELLANDER, S., Comparative studies on the adrenergic neuro-hormonal control of resistance and capacitance blood vessels in the cat. *Acta physiol. scand.* 1960. *50*. Suppl. 176.

MELLANDER, S., Comparative effects of acetylcholine, butyl-nor-synephrine (Vasculat), noradrenaline and ethyl-adrianol (Effontil) on resistance, capacitance and precapillary sphincter vessels and capillary filtration in cat skeletal muscle. *Angiologica* 1966. *3*. 77—99.

PAPPENHEIMER, J. R. and A. SOTO-RIVERA, Effective osmotic pressure of the plasma proteins and other quantities associated with the capillary circulation in the hindlimbs of cats and dogs. *Amer. J. Physiol.* 1948. *152*. 471—491.

RENKIN, E. M. and S. ROSELL, Effects of different types of vasodilator mechanisms on vascular tonus and on transcapillary exchange of diffusible material in skeletal muscle. *Acta physiol. scand.* 1962. *54*. 241—251.

ROSELL, S. and B. UVNÄS, Vasomotor nerve activity and oxygen uptake in skeletal muscle of the anesthetized cat. *Acta physiol. scand.* 1962. *54*. 209—222.

Acta physiol. scand. 1968. 72. 157—171

From the Institute of Neurophysiology, University of Oslo, Norway

Nervous Control of Urinary Bladder in Cats

I. The collecting phase

By

PER EDVARDSEN

Received 31 May 1967

Abstract

EDVARDSEN, P. *Nervous control of urinary bladder in cats. I. The collecting phase.* Acta physiol. scand. 1968. 72. 157—171.

Intravesical pressure under isometric conditions was studied in anesthetized cats after operative canalization of the bladder. Records were obtained after selective transection at the peripheral and at the intraspinal level of the sympathetic and parasympathetic nerves supplying the bladder, after rhizotomy of the thoraco-lumbar and sacral nerves to the bladder, and after transection of the spinal cord at various levels. With small bladder volumes no change in pressure was observed after the various lesions, but as the volume increased, the concomitant rise in bladder pressure was progressively inhibited by sympathetic nervous impulses. In particular was this inhibition marked during the last one-third of the cystometrogram. The sympathetic influence is maintained by a spinal reflex with the afferent path through the posterior sacral roots, its stimulus being stretching of the bladder wall. The effect of this spinal adaptation reflex is thus to permit increasing bladder volumes with only moderate increases in bladder pressure. Non-specific bladder responses consisted in an increase in motility caused by the laparotomy and an inhibition of motility caused by any intraspinal procedure. This inhibitory effect was found to be due to discharge through the sympathetic nerves on manipulation of spinal structures, regardless of their relation to the segments supplying the bladder.

The urinary bladder serves two opposing functions — those of collection and expulsion of urine. Of these two, that of collection is still the subject of conflicting opinions as regards the possible nervous mechanisms responsible for the maintenance of a low intravesical pressure during the storage of urine. The assumption of previous workers that this was either an adaptation mechanism involving a spinal parasympathetic reflex (Sherrington 1915), or reflex activity in the vesical plexus (Denny-Brown and Robertson 1933a, 1933b), or an accommodation effect due to inhibition from supraspinal levels (Langworthy and Kolb 1933, Langworthy and Hesser 1936), was discarded by Tang and Ruch in 1955. On the basis of supraspinal transection experiments in cats, the latter authors concluded that the bladder was not subject to nervous control during the collecting phase. Similar conclusions, based on clinical investigations, were reached by Nesbit and Lapides in 1948. More recently it was asserted by Ruch (1965) that the slope of the pressure-volume curve indicates that

the bladder follows the laws of Laplace and Hooke concerning elastic balloons on filling, which would suggest the operation of a non-neurogenic mechanism in the collecting phase.

On the other hand, Gjone (1965) concluded from the effects of section of the sacral parasympathetic nerves and the lumbar sympathetic ganglionic chains, that the bladder is subject to a tonic excitatory influence through its parasympathetic nerve supply and to a tonic inhibitory influence mediated by its sympathetic nerves. This dual nervous influence was suggested to be of supraspinal origin, as stimulation of cortical and subcortical areas resulted in bladder contraction only in the presence of intact parasympathetic nerves to the bladder while inhibitory responses were obtained only when the sympathetic nerve supply was preserved. Although the concept of a dual supraspinal influence on the bladder is compatible with the 'accommodation theory' of Langworthy *et al* it is hardly consistent with the assertion that bladder pressure is not influenced by brain stem transection at various levels (Tang 1955, *Tang and Ruch 1955 Plum and Colfelt 1960*)

The present series of experiments was initially designed to investigate the long-term effects of selective bladder denervation. However as certain observations were difficult to reconcile with the non nervous theory of Ruch as well as the dual autonomic influence proposed by Gjone, the transection experiments of these authors were repeated and extended so that the immediate as well as the long-term effects were observed and these using various bladder volumes.

Earlier workers have used the term 'bladder tone' synonymously with 'muscular tension of the bladder wall' But the 'tone' also encompasses several other qualities, such as the elastic tension in the bladder wall the hydrostatic pressure within the bladder the hydrodynamic properties of the fluid, the resistance in the recording paratus etc. In the present report, therefore the term 'bladder tone' will be used in accordance w th the operational definition gi en by Ruch (1960) i.e. the slope of the pressure olume curve obtained by cystometry

Material and methods

The result are based on data obtained from experiments on 55 cats, mostly males, weighing from 1.6 to 5 4 kg

As general anesthetic pentobarbital sodium (Nembutal® Abbott) was administered intraperitoneally in doses of 30 mg/kg body weight initially with small incremental intramuscular doses as required The animal lay on heated pad controlled by thermostat.

After laparotomy plastic catheter of 3 mm bore was inserted by suprapubic approach into a slit in the middle part of the exposed rethra, and so introduced into the bladder T prevent leakage ligature was passed around the rethra and the catheter A three-way tap wa ttached to the latter and was then in turn connected to the filling syringe nd to Statham pressure transducer for isometric recording on an ink-writing Grass Type 5C polygraph The bladder content was replaced by saline t body temperature

The parasympathetic pelvic nerves were reached through the same abdominal incision and isolated in a loop of nylon thread prior to the introduction of the catheter The later section of the nerves, which was performed proximal to their ramification, could then be achieved with minimum exposure of the bladder and bdominal viscera, as only the lowermost part of the incision had to be reopened

Interruption of the sympathetic erve supply was effected by section of the hypogastric nerves and extirpation of the inferior mesenteric ganglia This procedure was lso performed through

the same abdominal incision after reopening though of necessity it caused more extensive exposure and manipulation of the bladder than did section of the pelvic nerves.

Neurotomy and rhizotomy of the spinal nerves, and transection of the spinal cord were preceded by laminectomy to the required extent before any bladder recordings were made. The dura was then covered by moist pads and the wound closed until the procedure proper was started. As the measured intravesical pressure was influenced by the animal' position, this latter was maintained constant throughout recordings made before and afte any one of th nerve sections. Recordings made before and after section of the pelvic and hypogastric nerves were obtained with the animal in the supine position, while for those before and after intraspinal procedures, the animal lay prone. I the latter instance the distal part of the animal was suspended by book to prevent undue pressure on the bladder.

The blood pressure was measured in the femoral artery and transferred to the polygraph, as were the thoracic respiratory excursions. Open airways were maintained by the insertion of an intratracheal cannula. In long-lasting operations sol tions of 5 % glucose/0 9 % saline were administered via either the external jugular or the femoral ein.

Results

(1) *Non specif c Side Effects*

The majority of the experiments performed in the present study involved rather ex tensive surgical procedures, and it is reasonable to suppose that these may induce changes in the activ ty of an excitable organ such as the bladder. It was found that bladder motility is also influenced by a number of external factors such as the magnitude of the volume increments, the frequency and velocity of filling the temperature of the instilled saline, etc.

Keeping the external conditions as far as possible constant three important causes of non-specific changes in bladder motility were found during the course of the experiments, and since they have an important bearing on the interpretation of the f nal results, they will be dealt with first.

(a) *Effects f laparot my* (4 expts.) Test operations were carried out to assess the effects of exposing the bladder to air and to manipulations similar to those actually performed when the peripheral nerves to th bladder were transected transperitoneally. The results from one such operation are shown in Fig 1 namely rise in bladder pressure and an augmentation of the spontaneous contractions when the bladder olume was kept constant. At the same time, the micturition threshold was lowered, and in fact when laparotomy was performed using larger bladder volumes, the latter had to be reduced to a small fraction to prevent micturition.

The increased bladder motility following laparotomy was most marked immediately after closure of the abdominal incision and decreased gradually thereafter In one experiment, however some effect was still present after 1 hr Furthermore, the magnitude of the effects appeared to be in direct proportion to the degree of bladder exposure. Thus, following an operation simulating section of the pel ic nerves, with exposure of only the urethra and adjacent distal part of the bladder the bladder reaction was much less than in a sham-sympathectomy operation involving exposure of the whole organ. Finally the effect was greatly enhanced by the collection of blood in the abdominal ca ity and in one case in which this occurred no tendency to normalisation of bladder motility was detectable even after 4 hrs of observation.

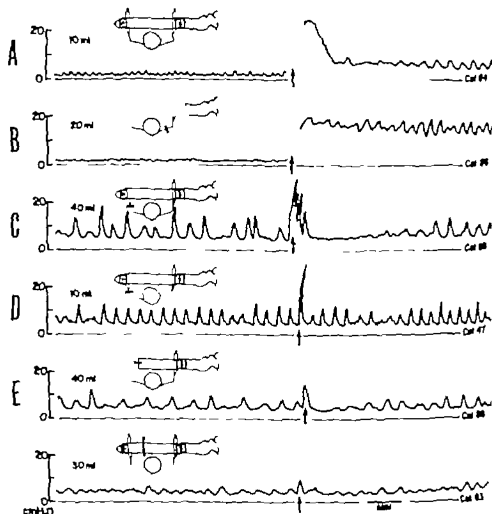


Fig 1 Non-specific effects. Insets show site of lesion. Bladder volume indicated to the left. Actual procedure performed at arrows. Effect of laparotomy in the intact animal (A) and of additional section of the hypogastric nerves after previous central denervation of the bladder (B). Effect of sectioning the sacral nerves in the presence (C) and absence (D) of sympathetic nerves to the bladder. Effect of pinching the cord after central parasympathetic denervation (E) and of transecting the cord after bladder denervation (F)

In two additional experiments, the hypogastric nerves were sectioned after previous extirpation of the spinal cord below Th_{1}, i.e. after central denervation of the bladder. The effect of this procedure on bladder pressure was essentially similar to that obtained by laparotomy alone in the intact animal (Fig 1 B)

(b) *Effects of intraspinal operative procedure* (7 expts.) All intraspinal procedures caused a temporary depression of bladder activity, i.e. a decrease in pressure and spontaneous contractions and an abolition of the micturition reflex. The depression lasted from some minutes to several hours, usually for 10—30 min. Although also

observed after laminectomy alone it was most clearly seen in procedures which involved manipulation of subdural structures (Fig 1 C) and furthermore the general impression was that the effect was more marked after interference with the lower part of the spinal cord than with the upper

This bladder depression also occurred when the pel ic nerves were sectioned prior to the intraspinal operations, but, on the other hand, it was never encountered after a previous section of the sympathetic nerve supply whether this was performed immediately or days before the intraspinal procedure (Fig 1 D) Moreover after removal of the sacral segments of the cord, i.e of the parasympathetic outflow to the bladder a depression could be produced by pinching the proximal sectioned end of the cord with a pair of forceps (Fig. 1 E) On the other hand, transection of the lumbar portion of the cord had no obvious effect on the motility of the extrinsically denervated bladder (Fig 1 F) indicating that adrenals activated by the intraspinal procedures play a minor part in the depression of the bladder It was concluded therefore, that this observed effect of bladder inhibition is brought about by the sympathetic nerves.

(c) *Effects of th an mal's posit on* (2 expts.) It was found that the bladder pressure and spontaneous contractions varied in a haphazard way with alterations in the animal' position, i.e from supine to prone or to lying on its side. There is no evidence in the literature that the tension in smooth muscle is influenced by changing the position of the subject, as is the case in the somatic system. A major cause of these ariations is probably changes in the hydrostatic pressure in the recording system due to the different positions in the abdomen taken by the bladder

In consequence of these observations it was necessary to allow at least one hour to elapse after th insertion of the bladder catheter or after any other intra-abdominal procedure before recordings were taken. Furthermore, in experiments involving nerve transection, tonic nervous influences were likely to be more reliably recorded with the animal in one position and using a constant bladder olume than by cystometrograms. Finally it was necessary to take into account in intraspinal operations the depressive effects exerted through the sympathetic nerves.

( ) *Sectio f the Parasympathetic N rve Supply*

Section of the parasympathetic nerves to the bladder abolishes the micturition reflex. In the intact animal, on the other hand, the approximate olume threshold for mic turition may be adduced from the cystometrogram by means of two fairly constant features (i) the spontaneous contractions, which become isible at bladder olumes between one-third nd one-fifth of the micturition threshold and thereafter increase in frequency and diminish in amplitude until micturition occurs, and (ii) the ppear ance of segment III This is the name given to the final sharp rise in the hitherto flat pressure olume curve which indicates that the bladder wall is becoming less compliant to the increasing volume and which thus precedes the micturition con traction.

As peripheral and intraspinal parasympathetic transection had different effects, they will be dealt with separately

(a) *Peripheral s ction* (8 expts.) The immediate effects of sectioning the pelvic nerves were a rise in bladder pressure and an augmentation of the spontaneous contractions. Th only apparent difference between these effects and those obtained in the test laparotomies mentioned above was the loss of micturition contractions following section of the pelvic nerves while micturition was preserved when these latter were intact. A decrease in spontaneous contractions and bladder pressure was never encountered after pelvic nerve section. Effects due to different bladder volumes after pelvic nerve section were difficult to assess owing to the additional variations caused by the laparotomy

(b) *Intraspinal sect on* (14 expts.) The immediate effects of sectioning the sacral nerves intraspinally or of any other intraspinal procedure, were a decrease in bladder pressure and in spontaneous contractile movements. After the gradual disappearance of this initial depression, which usually lasted for some 10—30 min, the subsequent effects depended on the bladder volume relative to the micturition threshold. Thus at bladder volumes below about one-third of the micturition threshold value no significant effect on the bladder was observed. But at volumes greater than two-thirds of the bladder capacity an increase in intravesical pressure usually occurred (Fig 2 A)

In 6 additional experiments, selective transections of the posterior sacral roots were performed The effects of these operations were essentially the same as those obtained by section of the sacral nerves (Fig 2 B) Selective transection of the ventral sacral ots (3 expts.) with or without additional sections of the dorsal roots had no effect on the pressure (Fig 2 C)

When the sympathetic nerves were cut prior to intraspinal transection (6 expts.) whether of the sacral nerves (Fig 1 D) or of their ventral or dorsal roots, no alteration in the pressure within the bladder occurred, regardless of the contained volume This negative ffect was observed whether the sympathectomy was performed immediately or days before the intraspinal operation.

These last findings described above give strong support to the hypothesis that the bladder is not subject to sustained parasympathetic influence during the collecting phase (see introductory part) This is corroborated by the fact that selective transection of the sacral ventral roots had no effect on the pressure whatever the bladder volume. A further point in fa our of this theory is that a decrease in pressure and spontaneous contractions was never observed after section of the pelvic nerves, although any loss of parasympathetic excitatory influence might be masked initially by the transient effects of laparotomy The decrease in bladder pressure and spontaneous contractions following intraspinal parasympathetic nerve section reported by Gjone (1965) are shown by the present experiments to be non-specific effects dependent on an intact sympathetic nerve supply to the bladder Gjone reported also-

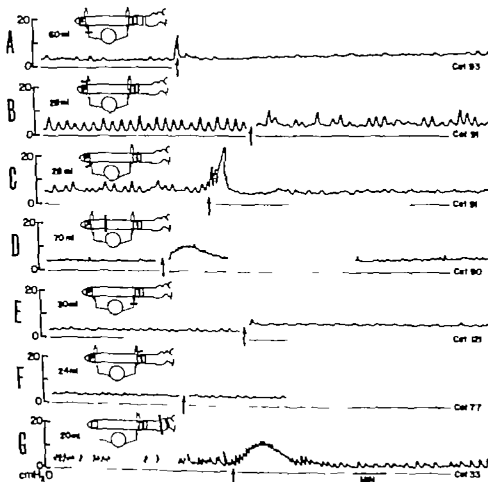


Fig. Effects on bladder pressure of intraspinal transections (↑) t various levels using large bladder volumes. Insets show site of lesion. After an initial depression the pressure rises after section of the pelvic nerves subsequent to high thoracic cord transection (A) fter section of the dorsal sacral roots (B) the lumbar cord (D) and the thoraco-lumbar spinal nerves E). The pressure is unchanged after section of the ventral sacral roots preceded by posterior rhizotomy L —S_4 (C) after section of the thoraco-lumbar dorsal roots (F) and after brain stem transection in decerebrated nimal with previously sectioned sacral nerves (G). The transient pressure levations immediately following the transections are caused by straining and twitching of the animal on the transection.

tical effects whether the entral or the dorsal roots or the sacral nerves themsel es were transected.

The different results of transection of the posterior sacral roots obtained with small and large bladder olumes suggest that afferent impulses from the bladder *via* the sacral nerves participate in the regulation of bladder pressure at larger volumes. The loss of this effect after sympathectomy indicates that the efferent impulses responsible for bladder inhibition are mediated through the hypogastric nerves.

(3) *Interruption of the Sympathetic Nerve Supply*

The sympathetic nerve supply to the bladder is derived from the cord segments Th_{12}—L_2 and reaches the bladder through the hypogastric nerves. Sympathectomy can thus be carried out *peripherally* or *intraspinally*

(a) *Peripheral section* (9 expts.) The immediate effect of section of the hypogastric nerves was similar to that of laparotomy *i.e.* an increase in bladder activity consisting of a rise in bladder pressure and augmentation of the spontaneous contractions leading to micturition. No significant difference was observed whether the operation carried out was a simple section of the nerves or whether it also included extirpation of the inferior mesenteric ganglia.

Since micturition occurred following laparotomy using large bladder volumes, whether or not the hypogastric nerves were cut, comparisons of the effects on pressure of varying degrees of bladder distension were not obtained. Nor could comparisons justifiably be made after abolition of the micturition reflex by previous section of the spinal cord (2 expts.) or of the parasympathetic nerve supply (3 expts.) because of the masking effects from the laparotomy. The effects of sympathectomy at large bladder volumes were, however, remarkably small after interruption of the parasympathetic nerve supply (2 expts.)

(b) *Intraspinal section* (3 expts.) To secure as far as possible complete interruption of the sympathetic outflow to the bladder, the spinal nerves were sectioned on both sides from Th_9—L_4. In two additional experiments the dorsal roots only from Th_7—L_4 were sectioned. The extensive laminectomy preceding the sections caused a profound depression of the bladder motility, as well as a deterioration in the animal's general condition. Usually some 4—5 hrs had to elapse before satisfactory experimental conditions as judged by the blood pressure, respiration and tendon reflexes were established.

In one experiment the spinal nerves were transected at a small bladder volume without any significant effect. In two experiments performed at large bladder volumes the section was followed by a rise in bladder pressure (Fig. 2 E). Selective section of only the posterior roots from Th —L_4 under similar conditions had no effect (Fig. 2 F).

Selective transection of the thoraco-lumbar ventral roots was not attempted, since this procedure would inevitably damage the cord.

The results of sympathetic nerve section set out above suggest that the inhibitory effect on bladder pressure is mediated through the ventral thoraco-lumbar roots, and that the dorsal roots of the corresponding segments have no part to play in this mechanism. Furthermore, the sympathetic inhibition appears to be dependent on the bladder volume, since a release of inhibition is seen only at volumes approaching the micturition threshold. The similarity between the effects of sympathectomy and ablation of the dorsal sacral roots is clearly demonstrated.

(4) *Transectio f the Spinal C rd and Brain Stem*

Since the effects on the bladder of sympathetic nerve section corresponded to those of sacral dorsal root section, the relationship between the afferent sacral and the efferent sympathetic nerves was likely to be elucidated by spinal cord transections at various levels. A mid-lumbar transection would interrupt the presumptive connection between the afferent sacral fibres and the sympathetic outflow to the bladder lea ing the latter subjected only to supraspinal influences. On the other hand a high thoracic transection would preserve the intraspinal connection between the afferent and efferent limbs at the same time removing the supraspinal influence on the sympathetic neurones. Finally brain stem transections might disclose any supraspinal tonic impulses influencing the inhibitory reflex.

(a) *Mid-lumbar t ansection* (12 expts.) The effect of transecting the cord at the level of L_3 was similar to that of section of the sacral nerves or of the dorsal sacral roots. Thus, with small bladder volumes, no changes were observed after an initial transient depression, while using larger volumes a rise in pressure was recorded (Fig. 2 D) The initial depression was often in part masked by a brief rise in pressure due to straining and twitching of the animal on cord section. The effect was essentially similar if the mid-lumbar transection was preceded by section of the cord in the upper thoracic segments (5 expts.) On the other hand, if sympathectomy (2 expts.) or sacral neurotomy (1 expt.) had been performed prior to the lumbar cord section, no effect was observed when using large bladder volumes.

(b) *High thoracic transection* (12 expts.) The level of transection was in 7 cases Th and in 5 cases Th_4. None of these transections affected the bladder pressure at any olume apart from the transient depression immediately after the procedure. Using large bladder volumes, an additional section at mid-lumbar level, however caused a rise in pressure. High thoracic transection was performed in 4 experiments after section of the sacral nerves or the lumbar cord, i.e with an interrupted para sympathetic but an intact sympathetic system. In none of these cases was there any significant change in bladder pressure with small or large olumes.

(c) *Brain tem t ansections* (7 expts.) Transection was performed t hypothalamic, supracollicular intercollicular and subcollicular levels, all of which procedures alter the olume threshold for micturition (Barrington 19?1 Tang and Ruch 1955 Edvardsen 1967b) In the absence of micturition, however the brain stem transections had no effect on the pressure within the intact (2 expts.) or sympathectomized (3 expts.) bladder In 2 experiments the transections were preceded by section of th sacral nerves, leaving the sympathetic system intact. Not under these conditions either was there any change in bladder pressure after the transections (Fig. 2 G)

The results of the high thoracic and brain stem transection experiments indicate that in the anesthetized cat there appears to be no tonic supraspinal influence on bladder pressure neither through the sympathetic nor through the parasympathetic

nervous systems. This is in agreement with the assumption made above that the parasympathetic sacral nerves exert no excitatory influence on the bladder during the collecting phase On the other hand the similar effects of mid-lumbar transection and section of the dorsal sacral roots and thoraco-lumbar spinal nerves seem to support the hypothesis that efferent sympathetic activity is evoked by afferent impulses coming by the sacral nerves. Finally the persistence of the sympathetic inhibitory effect after high thoracic transections indicates that the sympathetic outflow is initiated at the spinal level.

Since no effects were seen with smaller bladder volumes, the *general conclusions* which may be drawn from the transection experiments, are as follows (i) During the collecting phase the bladder is not subject to parasympathetic tonic influence. (ii) As the bladder volume is increased sympathetic inhibitory influences come into play with th effect of counteracting the rise in bladder pressure. (iii) The *sympathetic activity is evoked by afferent impulses mediated through the sacral dorsal roots.* (iv) Initiation takes place at spinal level.

(5) *Effects of Peripheral and Intraspinal T ansections on the Cystometrogram*

Although non-specific bladder reactions caused by external factors could be eliminated by recording at a constant bladder volume, some disadvantages of this method were conspicuous Firstly the effects of laparotomy which might last for 1 hr prohibited an estimation of the effects of section of the hypogastric or the pelvic nerves, since the bladder volume increased on account of the animal s production of urine during this time span Similarly an increase in bladder volume might cause some uncertainty if th non-specific bladder depression after the intraspinal procedure was long lasting The main objection to the method, however was the uncertainty in reading when the non specific spinal depression had passed off since individual variations were apparent and also the bladder recovery was gradual. It therefore seemed desirable to check the results obtained by cystometry comparing the preoperative cystometrograms with those obtained 2 or more hrs after the operation, i e after a period of time when the effects of laparotomy as well as the non-specific effects of the intraspinal operation were likely to have passed off The external factors such as injection velocity and frequency position of the animal, etc., were kept as constant as possible.

The preoperative cystometrogram (Fig 3 A) was recorded up to the micturition threshold, care being taken not to exceed this so as to avoid micturition contractions occurring against a closed outlet After emptying the bladder the actual lesion was inflicted, and then after evacuating th urine which had accumulated during the postoperative period a second cystometrogram was obtained (Fig 3 B) The slopes of the pressure volume curves of the two cystometrograms, defined operationally as bladder tone, were plotted and compared. each point on the plot representing the intravesical pressure 1 min after each filling (Fig 3 C) At this moment a steady level of intravesical pressure had been reached in the intact animals, but not in some of the operated ones. A striking feature of the operations affecting the bladder pressure as

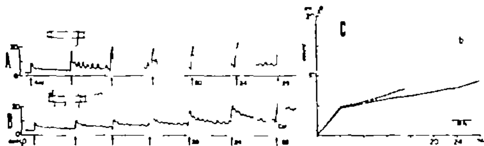


Fig. 3 Effects on the cystometrogram of sectioning the dorsal sacral roots. V lume increments indicated by arrows. (A) Preoperative control, (B) cystometrogram 2 hrs after section, (C) plots of the cystometrograms indicated by and b respectively

a prolongation of the time needed to obtain a steady pressure level after each volume increment, indicating that some regulatory mechanism had been interfered with.

If the nerve lesion affected the slope of th pressure volume curve at all, the effect became more pronounced as the bladder volume was increased. Dividing the olume arbitrarily into three equal parts, it appeared that while during the first one-third the two slopes differed only slightly and in the middle one-third moderately there was a marked difference in steepness in the last one third. Thus, in estimating the effects of the various lesions, the steepness of the pressure volume curve relative to the zero line was measured in the last one-third of the cystometrogram.

A summary of the changes in the cystometrograms after arious nerv lesions in 19 cats is given in Fig. 4 The upper schematic drawing indicates the sites of transection, the lower illustration is of the pressure olume curves obtained. As the micturition threshold (M) aried considerably they are all set t 3 3, and the rise in tone is measured for each individual from the point of 3 of the micturition threshold.

As judged by the rise in tone as the bladder filled, the cy tometrograms are seen to constitute two separat groups. The first group (A) falling in the chosen plot scale

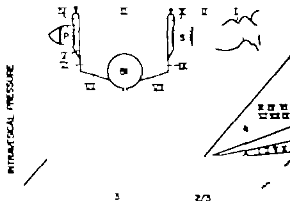


Fig 4 Schematic presentation of effects obtained on the cystometrograms after arious nerv lesions in 19 cats Upper drawing shows site of lesion lower drawing shows the rise in bladder pressure when filling the last one-third of volume before the micturition threshold (M) is reached Level of transections I—intercollicular II -high thoracic III—mid-lumbar IV—sacral dorsal roots V—sacral entral roots VI—sacral nerves VII—pelvic nerves VIII hypogastric nerves IX—thoraco-lumbar spinal nerves X—thoraco-lumbar dorsal roots.

between 5 and 12 degrees, includes the cystometrograms of all the intact animals (19 expts.) and those obtained in the transient period of depression immediately following intraspinal procedures (3 expts.) spinal cord transections cranial to the sympathetic outflow (5 expts.) brain stem transections (2 expts.) section of the ventral sacral roots (2 expts.) and section of the posterior roots of nerves supplying sympathetic fibres to the bladder (2 expts.) The second group (B) falling between 20 and 50 degrees, contains the cystometrograms of animals with sectioned hypogastric nerves (3 expts.) and intraspinal section of nerves supplying sympathetic fibres to the bladder after high thoracic cord transection (3 expts.) section of the pelvic nerves (2 expts.) section of the sacral nerves (3 expts.) or posterior sacral roots (6 expts.) and transection of the spinal cord between the points of emergence of the sympathetic and parasympathetic nerves to the bladder (2 expts.) Thus, the results of cystometry support the hypothesis of a spinal reflex mechanism causing inhibition of the bladder its afferent and efferent pathway being the dorsal sacral roots and the hypogastric nerves respectively The limits of the two groups would probably be narrower if the micturition threshold could be determined more accurately in the absence of the micturition reflex.

Of particular interest is the observation that the cystometrograms obtained during the period of bladder depression following intraspinal procedures resembled those obtained preoperatively both as regards flatness of the pressure/volume curve and the ability to obtain rapidly a steady pressure level after instillation of the volume increments. Since bladder depression was observed only when the sympathetic nerve -ply to this organ was intact, it appears that the non-specific sympathetic discharge d by the intraspinal operation and that caused by response to afferent bladder pulses coming *via* the sacral nerves of the intact animal, had similar effects, i.e. prevention of a rise in pressure. Previous experimental (Tang and Ruch 1955) and clinical (Nesbit *et al* 1947 Lapides 1953) investigators have reported that bladder tone remains unchanged during the period of spinal depression usually denominated as 'spinal shock' following operative interventions with the cord. But this observation was interpreted by these workers as proof of absence of neurogenic influence on the bladder during the collecting phase.

(6) *Observations in Decer b at d Cats*

It was shown that the features observed n the pentobarbital anesthetized cat are qualitatively similar to those obtained in the non-anesthetized animal. Thus, 6 experiments were performed in decerebrated preparations In four of these the operative procedure consisted of transection respectively of the upper thoracic cord, the mid-lumbar cord the posterior sacral roots and the thoraco-lumbar spinal nerves using large bladder volumes The other two experiments involved respectively section of the dorsal sacral roots and section of the hypogastric nerves using small bladder volumes. The effects of all these procedures were essentially similar to those obtained using anesthetized animals, both as regards the isometric recordings and the cystometrograms.

Discussion

The present experiments indicate that during the collecting phase the urinary bladder acts independently of influences from the central nervous system until a certain intravesical pressure is reached. From this moment on a further increase in pressure is counteracted by a spinal reflex mechanism having its afferent input through the sacral nerves and its efferent output through the sympathetic nerves.

A spinal reflex with its afferent pathway through the dorsal sacral roots implies the presence of tension receptors in the bladder wall. Such receptors were in fact demonstrated by Iggo (1955) who recorded afferent impulses in myelinated fibres of the pelvic plexus. When the bladder was empty no impulses were obtained. At small bladder volumes a few impulses were recorded, while with increasing volumes the impulse frequency reached a maximum rate of 20—30/sec, above which a further bladder filling had no effect. Iggo concluded that the bladder stretch receptors were situated in series with the muscle cells. Similar observations on afferent nerve activity were made by Talaat (1937) using the pelvic nerves. Afferent impulses from the hypogastric nerves were only obtained when the bladder was markedly overdistended. These impulses were presumed to convey pain.

Efferent sympathetic activity in the hypogastric nerves was studied by Marsh, Suzuki and Meyers (1959) who found that it decreased markedly when the pelvic nerves were sectioned. It was also reduced for 30—60 min after application of local anesthesia to the pelvic nerves, i.e. for as long as these nerves were blocked. The experiments were performed when the bladder was empty and no attempt was made to decide whether the reflex was a spinal or a supraspinal one. However, Coote and Downman (1966) have recently recorded postganglionic sympathetic impulses in the cardiac and renal nerves in response to stimulation of the dorsal spinal roots. Furthermore McPherson (1966) reported a diminution in the spontaneous activity of the bladder on stimulation of the lumbo-sacral dorsal roots in anesthetized cats. The conclusions arrived at above from the results of transection experiments would thus appear to be compatible with those obtained from electrophysiological investigations.

In a preliminary report (Edvardsen 1966) misinterpretation of the laparotomy effect led to the assumption of a sympathetic afferent pathway causing the bladder inhibition. In those experiments the author was not aware of the effects of laparotomy *per se* and the observations were made at a time when these effects predominated. Thus, the conclusion drawn was not confirmed by the present experiments. On the contrary, the lack of change in the cystometrograms after section of the posterior thoraco-lumbar roots indicates that afferent impulses from the bladder mediated through the hypogastric nerves do not participate in the regulation of its pressure which tallies with the observations made by Talaat (1937) when investigating sympathetic afferent activity.

A reflex arc with a sacral afferent and a sympathetic efferent arm might also account for the observations of Shuhito *et al.* (1964) who found that after section of either the hypogastric or the pelvic nerves there was a diminution in the O_2-conr

tion and the CO_2-production of the vesical wall as well as a diminution in the activity of succino-dehydrase and cholinesterase, while the activity of lacto-dehydrase and the speed of incorporation of P_{32} into phosphoric compounds were augmented. No such metabolic changes were observed after section of the ventral sacral roots, or after the additional section of the dorsal roots peripheral to the spinal ganglia.

A spinal inhibitory reflex on the bladder subserved by sacral afferents, provides possibly also an explanation for the hypertonic bladders observed by some investigators after section of the parasympathetic nerves (Carpenter and Root 1951 Langley and Whiteside 1951 Shihito 1961) This hypertonicity may be due in part to the loss of sympathetic reflex inhibition caused by the interruption of the afferent pathway and in part to the metabolic changes demonstrated by Shihito *et al.* (1964). Similar effects have been obtained in cats immediately (Gjone 1965) and some time (Elliott 1907 Barrington 1915) after sympathectomy

The presence of an inhibitory sympathetic influence on the bladder was also suggested by Gjone (1965) But, his assumption of a supraspinal origin for this effect has not been confirmed by the present experiments. The persistence of the sympathetic inhibition after high thoracic cord transection strongly indicates a spinal origin. Furthermore the loss of inhib tion after section of the cord caudal to the sympathetic outflow as well as after section of the sacral nerves or the posterior sacral roots shows that the mechanism is one of reflex spinal inhibition.

As shown by the present experiments, the sacral afferent → sympathetic efferent spinal reflex constitutes a negative feed-back affecting bladder tone. Interruption of it results n a steeper rise in the slope of the pressure/volume curve. It therefore appears that the effect of the sympathetic nervous stimulus to the bladder is to render sarcoplasmatic and myoplasmatic fibrils of the smooth muscle cells more compliant to increasing stretch This hypothesis is supported by observations made after stimulating the hypogastric nerves (Edvardsen 1967d) under isotonic conditions the bladder volume increased within certain limits almost proportionally to the stimulation frequency The theory put forward by Ruch (1965) of a purely mechanical basis of bladder tone control probably holds good for the small volumes following sympathectomy while sympathetic inhibitory control appears to allow for the bladder to follow the laws formulated by Laplace and Hooke at larger volumes as well.

Although the concept of a parasympathetic spinal reflex of adaptation (Sherrington 1915) as well as a similar plexus reflex (Denny-Brown and Robertson 1933a, 1933b) can no longer be accepted as also maintained by Tang and Ruch (1955) the present experiments still suggest the existence of some sort of reflex adaptation mechanism. However this spinal reflex ppears to be composed of th afferent pathway suggested by Sherrington and the efferent pathway of Denny Brown and Robertson.

References

BARRINGTON, F. J. F., The nervous mechanism of micturition. *Quart. J. exp. Physiol.* 1915 *8* 33—71

BARRINGTON, F. J. F. The relation of the hind-brain to micturition. *Brain* 1921 *44* 23—53

CARPENTER, F. G. and W. B. ROOT. Effect of parasympathetic denervation on feline bladder function. *Amer. J. Physiol.* 1951 *166* 686—691

COOTE, J. H. and C. B. DOWNMAN, Central pathways of some autonomic reflex discharges. *J. Physiol.* (Lond.) 1966 *183* 714—729

DENNY-BROWN, D. and E. G. ROBERTSON. On the physiology of micturition. *Brain* 1933a. *56* 149—190

DENNY-BROWN, D. and E. G. ROBERTSON, The state of the bladder and its sphincters in complete transverse lesions of the spinal cord and the cauda equina. *Brain* 1933b *56* 397—463

EDVARDSEN, P. Changes in urinary bladder motility following lesions in the nervous system in cats. *Acta neurol. scand.* 1966. *42* Suppl. 20 23—31

EDVARDSEN, P. Nervous control of urinary bladder in cats. IV. Effects of autonomic blocking agents on responses to peripheral nerve stimulation. *Acta physiol. scand.* 1968d. *72* 231—247

ELLIOTT, T. R. The innervation of the bladder and urethra. *J. Physiol.* (Lond.) 1907 *35* 367—445

GJONE, R. *A Dual Peripheral and Spinal Autonomic Influence on the Urinary Bladder* Oslo, Universitetsforlaget 1965.

IGGO, A. Tension receptors in the stomach and the urinary bladder. *J. Physiol.* (Lond.) 1955 *128* 593—607

LANGLEY, L. L. and J. A. WHITESIDE, Mechanism of accommodation and tone of urinary bladder. *J. Neurophysiol.* 1951 *14* 147—152.

LANGWORTHY, O. R. and F. H. HESSER, An experimental study of micturition released from cerebral control. *Amer. J. Physiol.* 1936. *115* 694—700

LANGWORTHY, O. R. and L. C. KOLB, The encephalic control of tone in the musculature of the urinary bladder. *Brain* 1933. *56* 371—382.

LAPIDES, J. Observations on normal and abnormal bladder physiology. *J. Urol.* (Baltimore) 1953 *70* 74—83

MALLIANI, D. J. G. STURLA and F. H. MEYERS, Role of afferent activity from the bladder in regulating its activity. *Amer. J. Physiol.* 1959 *196* 351—353

McPHERSON, A. The effects of somatic stimuli on the bladder in the cat. *J. Physiol.* (Lond.) 1966. *185* 185—196.

NESBIT, R. M., J. LAPIDES, W. W. VALK, M. S[illegible], R. L. BERRY, N. H. L[illegible], K. N. CAMPBELL and G. K. MOE, The effects of blockade of the autonomic ganglia of the urinary bladder in man. *J. Urol.* (Baltimore) 1947 *57* 21—230

NESBIT, R. M. and J. LAPIDES, Tonus of the bladder during spinal shock. *Arch. Surg.* 1948. *56* 138—144

PLUM, F. and R. H. COLFELT. The genesis of vesical rhythmicity. *Arch. Neurol.* (Chic.) 1960 *2* 487—496.

RUCH, T. C. Central control of the bladder. In *Handbook of Physiology*. Baltimore. Williams and Wilkins Co. 1960. pp. 1207—1223

RUCH, T. C., The urinary bladder. In Ruch and Patton, *Physiology and Biophysics*. Philadelphia and London. W. B. Saunders Comp. 1965 pp. 1010—1021

SHERRINGTON, C. S., Postural activity of muscle and nerve. *Brain* 1915 *38* 191—234

SHIMOTTO, S. Experimental studies on the innervation of the urinary bladder. *Urol. int.* (Basel) 1961 *12* 254—269

SHIMOTTO, S. SAITO, K. IWABA, A[illegible], N. NAKANO, Y. SHIRAI[illegible] and M. ARAI. The role of the spinal ganglion of the sacral nerve on the function of the urinary bladder. *Acta Neurochir.* (Wien) 1964 *25* 435—452.

TALAAT, M. Afferent impulses in the nerves supplying the urinary bladder. *J. Physiol.* (Lond.) 1937 *89* 1—13.

TANG, P. C. Levels of brain stem and diencephalon controlling micturition reflex. *J. Neurophysiol.* 1955 *18* 583—595

TANG, P. C. and T. C. RUCH. Non-neurogenic basis of bladder tonus. *Amer. J. Physiol.* 1955 *181* 49—57

Acta physiol. scand. 1968 72 172—182

From the Institute of Neurophysiology University of Oslo, Norway

Nervous Control of Urinary Bladder in Cats

II. The expulsion phase

By

PER EDVARDSEN

Received 31 May 1967

Abstract

EDVARDSEN P *Nervous control of urinary bladder in cats II The expulsion phase.* Acta physiol. scand 1968. *72* 172—182

The volume threshold for micturition is altered by transection of the brain stem at various levels and also by section of the sympathetic nerves to the bladder The aim of the present experiments has been to investigate whether the supraspinal influences are brought about by means of changes in peripheral sympathetic mechanisms. Mostly male cats were investigated using the direct cystometry method Cystometrograms obtained preoperatively were compared with those obtained after peripheral sympathectomy and during the phase of increased motility following laparotomy Then the effects of brain stem transection on the micturition threshold in the presence and absence of sympathetic innervation were compared. The peripheral factor responsible for eliciting the micturition reflex was found to be the tension in the bladder as assessed from the intravesical pressure The lower volume threshold found after sympathectomy is due to the hypertonicity of the bladder following this procedure The tonic supraspinal influence on the micturition reflex, on the other hand, was not altered by interruption of the sympathetic nerve supply to the bladder Thus, the expulsion of urine is controlled by two different mechanisms, namely peripheral regulation of the tension in the bladder wall and supraspinal regulation of the micturition reflex threshold.

The bladder volume at which the micturition reflex is initiated is altered by various brain stem transections (Tang 1955) These changes occur without concomitant changes in the cystometrogram (pressure/volume curve) (Tang and Ruch 1955 Edvardsen 1967a) and may accordingly be attributed to alteration of the reflex threshold. The micturition threshold in terms of bladder volume is also changed after ablation of the sympathetic nerve supply to the bladder (Elliott 1907 Barrington 1916, Gjone 1965) To what extent this is a consequence of the altered pressure/volume curve of the sympathectomized bladder (Gjone 1965, Edvardsen 1967a) or whether there is also an alteration of the reflex threshold for micturition has not been established. The primary object of the investigations reported here is to clarify this. Since the sympathetic influence on bladder motility was not taken into account by Tang (1955) it was necessary to compare the changes in micturition threshold after brain stem transections in preparations with and without an intact sympathetic nerve supply to the bladder

Material and methods

The results reported in this paper were obtained from experiments with 36 cats weighing between 1.8 and 5.4 kg of which 30 were males. The methods used were similar to those reported in previous article (Edvardsen 1967).

Pentobarbital sodium (Nembutal® Abbott) was given intraperitoneally in doses of 30 mg/kg initially supplemented thereafter by small intramuscular doses as necessary. In the decerebration experiments the animal was placed in head-holder in the prone position. The weight of the lower part of the body was taken by a hook suspended from cross-bar to avoid undue pressure on the bladder. After craniotomy the occipital lobe and the adjacent parts of the temporal lobes were removed by suction until the colliculi became visible and then the transections were performed using blunt spatula. When performed under ether anesthesia no bladder recording was undertaken for at least 45 mins after the decerebration in order to allow for elimination of the anesthetic. At the end of the experiment, 10 per cent solution of formalin was infused via the carotid arteries after opening the ... , the animal was decapitated, and the brain was fixed in situ by formalin for post mortem verification of the transection level.

After laparotomy the bladder was cannulated in the vertex region for direct cystometry (Murphy and Schoenberg 1960) employing the modification of this technique for experimental use (Gjone and Setekleiv 1963). A plastic catheter of 4 mm bore was connected by 3-way tap to the filling syringe and to the pressure transducer (Statham P23AC) changes in which were recorded on an ink-writing polygraph (Grass Model 5C). Thus the pressure was recorded after each volume increment of 2—4 ml saline by turning the tap to the appropriate position.

After reopening the abdominal incision, sympathectomy was then performed by means of section of the hypogastric nerves, removal of the inferior mesenteric ganglia, and extirpation of the lumbar ganglionic chains bilaterally. The abdominal cavity was closed again after this procedure.

Results

To avoid the non-specific effects on the bladder described in the previous article (Edvardsen 1967a) the following precautionary measures were taken. (1) Since bladder motility is markedly increased by the laparotomy per se and this effect can last for as long as up to 1 hr, no experimental procedures were carried out until at least 1 hr had elapsed after laparotomy. (2) The transient inhibition of bladder motility due to increased sympathetic activity following intraspinal operative procedures was taken into account when reading the recordings. (3) The position of the animal was kept constant during an experiment, as were other external factors such as velocity and frequency of filling, and the temperature of the saline instilled.

I Peripheral factors influencing micturition

In this series of experiments, 15 cats were examined cystometrically under pentobarbital anesthesia, and two after decerebration. The latter gave essentially similar results to those obtained from the anesthetized preparations.

From 36 preoperative direct cystometrograms obtained in the anesthetized animals, it appeared that the volume threshold level for micturition was fairly constant in each individual animal. The volume varied more with the depth of anesthesia than with the number of times the procedure was repeated, the maximum value being ± 10 per cent. In the decerebrated animals the micturition reflex was elicited at identical bladder volumes however often the experiment was repeated. Moreover in each individual animal the spontaneous rhythmic bladder contractions appeared

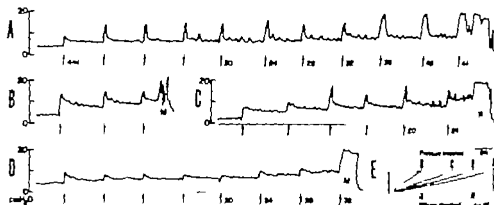


Fig. 1 Effects of laparotomy and interruption of the sympathetic nerve supply on bladder motility Supracollicular decerebration. Cystometrograms obtained preoperatively (A) immediat ly after the procedure (B) 30 min later (C) and 3 hrs later (D) Schematic diagram (E) indicates the licitation of micturition t identical levels of pressure, although at volumes.

at identical volumes and changed in a similar manner as micturition was approached. Finally the slopes of the pressure/volume curves — the tone — were the same on repeated examinations. The results thus indicate that, in the intact bladder there exists an interrelationship between spontaneous contractions, tone and the volume at which the micturition reflex is initiated.

The bladder motility was further investigated after altering the sympathetic outflow to this organ, the former experiments serving as controls. Repeated ... etric examinations were performed in 15 cats after peripheral section of the ...pathetic nerves (14 anesthetized — one decerebrated) and in 2 cats during ...essive sympathetic outflow caused by intraspinal procedures (one anesthetized — one decerebrated)

In the 15 sympathectomized cats a total of 52 cystometrograms were obtained at intervals ranging from a few minutes to 17 days after the sympathectomy operation. The general pattern of these experiments is illustrated in Fig. 1 It appears that the micturition reflex n the intact animal (A) is elicited at a bladder volume of 44 ml. Immediately after laparotomy and section of the sympathetic nerves this level was reduced to 12 ml (B) Half an hour later the volume threshold had increased to 24 ml (C) and 3 hrs after sympathectomy it had reached its maximum of 34 ml (D) which thus represented the final effect of the sympathetic denervation on the volume threshold for micturition. The micturition contractions were however initiated on each occasion when the intra vesical pressure exceeded 10 cm of water.

In the two experiments with increased sympathetic outflow to the bladder obverse effects were obtained (Fig. 2) The first cystometrogram (A) shows bladder motility after a laminectomy extending from the 6th thoracic to the 5th lumbar vertebra. The sympathetic inhibition is indicated on the cystometrogram by the delayed appearance of spontaneous contractions, the flatness of the pressure/volume



Fig 2 Effects of increased sympathetic outflow caused by laminectomy Supracollicular decerebration. Cystometrograms obtained during marked spinal depression (A) and 1 h later (B) ith shift of the micturition volume threshold to the left. Schematic diagram (C) show micturition at identical intravesical pressures

curve, and a high micturition volume threshold. The second cystometrogram (B) was obtained one hour later when, according to previous experiments (Edvardsen 1967a) the sympathetic effect is considerably diminished or even completely over The threshold for the appearance of spontaneous contractions and the micturition threshold were shifted to the left, and the slope of the pressure/volume curve was steeper The micturition contraction, however was initiated at the same intravesical pressure.

The recorded pressure thresholds for micturition varied between 10 and 15 cm H_2O in the group as a whole, but the value in any one cat was not altered by sympathetic denervation. The variability of the recorded pressures may in part be attributed to a different transducer setting in the various experiments (Edvardsen 1967a) but nevertheless suggests individual variations in the micturition reflex threshold. Comparing cystometrograms obtained on different days from the same animal the effect of a possibly different transducer setting was nullified by drawing an arbitrary zero line at the first volume increment and using this as a base-line for the subsequent intravesical pressure measurements.

These experiments suggest that the pressure threshold for micturition is not changed by altering the sympathetic outflow to the bladder Similarly the bladder tension receptors, messages from which initiate micturition, re not influenced by sympathetic activity Moreover the consta t initiation of th reflex t the same pressure level on repeated cystometric examinations demonstrates that th tension the bladder wall in terms of intravesical pressure is the principal peripheral factor responsible for the initiation of micturition.

It has been shown previously (Edvardsen 1967a) that interruption of the sympathetic nerve supply to the bladder is followed by steeper slope f the pressure/volume curve defined operationally as the bladder tone (Ruch 1960) The relationship between the tone and the volume thresholds for micturition is shown schematically in Fig 1 E and 2 C It appears that the steeper the slope the smaller the bladder volume at which micturition occurs and, conversely the flatter the slope the greater the volume threshold Thus the effect f sympathectomy on the volume threshold for micturition appears to be secondary to its effect on the



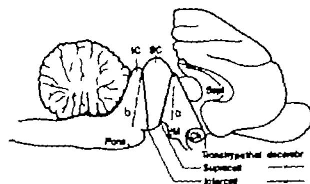


Fig 3 Upper part, medial level transection levels. Lower part, drawing of transection levels. SC and IC — superior and inferior colliculi. Ch — optic chiasm. M — mammillary corpus. a — transection level in cat 111. b — transection level in cats 70 and 71.

vesical pressure and the decrease in bladder capacity said to follow this operation (Elliott 1907 Barrington 1916, Langworthy Reeves and Tauber 1934 Gjone 1965) may be ascribed to an increase in bladder tone.

VII Central factors influencing micturition

In this series 15 cats were used. The investigation aimed to repeat the decerebration and brain stem transection experiments of Tang (1955) in two groups of animals, namely 8 intact and 7 previously sympathectomized cats. However since direct cystometry meant laparotomy the pre-decerebration micturition threshold was determined for 3 animals in each group under pentobarbital anesthesia. The rest of the experiments were carried out after decerebration performed under ether anesthesia, allowing time for the anesthetic to wear off first.

The levels of transection are shown in Fig 3. Using Tang's terminology the levels are called transhypothalamic supracollicular and intercollicular respectively. A subcollicular transection abolished the micturition reflex, as previously stated by Barrington (1921).

As there was no difference in the general pattern of response (i.e. blood pressure and respiration) to transection in the two groups, both are included in the general description of the experiments. The effect on the micturition volume threshold of the various transections are seen in Table I. As stated in a previous paper (Edvardsen 1967a) none of the transections altered the bladder tone.

TABLE I Micturition thresholds (ml) before and after brain stem transections in intact and sympathectomized animals

Cat no.	Weight g	Anesth.	A (I tact)	B (Transhyp.)	C (Supracoll.)	D (I tercoll.)
(I) Intact group						
7	3 400	Nemb.	30	14	24	14
114	4,200	—	100	70	70	34
115	4 600	—	44	24	84	24
107	2,650	Ether		10	26	16
110	4 600	—		10	38	14
111	2,600	—		—	16	6
113	3,500	—		16	44	24
118	4,900	—		12	100	16
Mean threshold ratios $\bar{A}/B$ = 1.80				$\bar{B}/C$ = 0 43		$\bar{C}/D$ = 2.80
Standard deviation S D = 0.36				S.D. = 0.29		S.D = 1.53
Significance P(< 1) = 0.01				P(> 1) = 0 02		P(< 1) = 0 12
(II) Sympathectomized group						
71	1,800	Nemb.	10	3	6	—
72	3,800	—	34	28	28	14
73	3,125	—	8	6	6	2
17	3,400	Ether		4	12	8
69	3,300	—		16	24	8
70	2,500	—		2	16	—
117	4,200	—		12	28	4

(1) T anshypothalamic t ansect o

The direction of this proximal transection was from just bove the anterior border of the superior colliculus to the hypophyseal groove

When performed under pentobarbital anesthesia, no behavioural effects were observed. However when performed under ether anesthesia, the transection was followed by marked changes in the rumal behaviou as the anestheti wore off, usually within 30 minutes. These consisted of marked hyper reactivity to external stimuli and an increased motor ctn ty closely resembling the sham-rage reaction (Bard 1950) recently discussed by Kaada (1967) Of particula interest was the observation that this reaction which seemed to be haracterized by generally enhanced sympathetic activ ty was accompanied by lowered micturition thresholds.

In the barbiturate nesthetized cats of both groups the mict rition threshold wa lowered (Table I) but the 'sham-rage did not occur In one animal, decerebrated under ether anesthesia (Cat 111) the proximal transection was placed caudal to the intended level (Fig 3) In this animal neither sham-rage nor a part ly low micturition threshold were observed.

(2) Supracollicular transection

This transection had the same point of origin as the transhypothalamic, but was angled more steeply so as to reach the ventral surface just caudal to the mammillary bodies.

In the pentobarbitalized cats no general effects of the transection were observed. In the animals decerebrated under ether anesthesia, on the other hand, the barrage was abolished instantaneously and in some of the animals a slight rigidity of the limbs ensued.

In most experiments the effect on the micturition was a rise in the volume threshold in both groups (Table I) and in one animal (Cat 115) even the pre-decerebration threshold was markedly exceeded. However although the heightening of the micturition threshold was conspicuous in all the unanesthetized animals, the effect was absent in 3 of the cats anesthetized with pentobarbital, one of these (Cat 114) being in the intact group and two (Cats 72 and 73) in the sympathectomized group. On post mortem examination the transections were found to be at the intended level in these cases.

(3) Intercollicular transection

This transection plane was nearly parallel with the last one emerging on the ventral surface just above the pons.

The operation did not affect the general condition of the barbiturate anesthetized cats, but in the unanesthetized animals, initially decerebrated under ether anesthesia, a marked rigidity of the limbs occurred, sometimes accompanied by opisthotonus. In both groups the transection lowered the micturition threshold (Table I). However in two animals in the sympathectomized group (Cats 70 and 71) the micturition reflex was abolished, the transection in these cases being through the anterior pons (Fig. 3 b).

As seen in Table I the volume thresholds for micturition in both groups were influenced by the transections. In the intact group the probability value (P) calculated by the t test indicates that it is unlikely that the observed changes are obtained by chance. The reduced significance value of the C/D-ratio (mean thresholds after supracollicular transection/mean thresholds after intercollicular transection) is supported by the statement of Tang (1955) that the mean threshold change produced by his corresponding transections was significant below the 1 per cent level.

Further it appears that the micturition thresholds in general in the sympathectomized group were lower than those in the group of intact animals. This was to be expected from the preceding experiments on peripheral factors influencing micturition. However calculating the ratios of the mean micturition thresholds after the transections, i.e. A/B, B/C, and C/D in each of the two groups, they were found to be 1.6—1.41, 0.45—0.59 and [illegible]7—2.35 respectively. This seems to indicate that the micturition thresholds were affected similarly by the transections in the

intact and the sympathectomized groups, although those in the latter were generally lower. From this observation it may be postulated that the supraspinal influence on the micturition threshold is essentially independent of the sympathetic nerves.

III Additional observations on spontaneous contractions and tone

In the intact animal spontaneous rhythmic bladder contractions appeared in the cystometrogram at volumes usually between one-fifth and one-third of the micturition threshold. As the volume increased further there was a gradual increase in contraction frequency until micturition occurred. The amplitude of contractions, on the other hand, also showed an initial increase but were then maintained at a constant level during the intermediate stages of bladder filling, finally decreasing gradually as the bladder volume approached the micturition threshold. Similar observations have been made by Plum and Colfelt (1960).

However, in accordance with the findings of Plum and Colfelt (1960), it was found that the spontaneous contractions also varied with the degree of bladder fullness in a similar manner after denervation of the bladder (expts.). It would thus appear that the change in the parameters of the spontaneous contractions due to bladder filling does not necessarily imply the existence of a nervous regulating mechanism, but merely demonstrates the effects of increasing tension on the spontaneous contraction pattern of smooth muscle.

Fig. 1 shows that laparotomy and section of the sympathetic nerves to the bladder were followed by increased activity of this organ consisting in augmented bladder contractions, hypertonicity and a lowered micturition threshold. As the immediate effects subsided, the appearance of the contractions on filling was delayed, the slope of the pressure/volume curve became flatter and the micturition threshold was shifted to the right. However, on comparing the spontaneous contractions appearing in different recordings at the same intravesical pressure, i.e. that of approximately 5 cm H_2O, they showed no appreciable variation at vesical volumes of 36 ml (A), 4 ml (B), 8 ml (C) and 24 ml (D). Thus it seems that, like the initiation of micturition, the parameters of the spontaneous contractions also are determined by the tension in the bladder wall. Such a relationship between spontaneous contractions and bladder pressure was postulated by Stewart (1900) who suggested further that the amplitudes were inversely proportional to the pressure, and that the muscle cells carried on the same amount of work on each contraction. It therefore seems unlikely that the spontaneous contractions are influenced separately without a concomitant change in bladder pressure, which even has been described recently by Lewin and Porter (1965) as occurring on stimulation of the globus pallidus.

Plum and Colfelt (1960) investigated the relationship between the appearance of spontaneous contractions and the occurrence of micturition. They found that if the threshold for spontaneous contractions was lowered by the induction of a chemical cystitis, the micturition volume threshold was also lowered. Conversely, on distension of the bladder the threshold for the appearance of spontaneous contractions was shifted to the right, and the micturition threshold was raised correspondingly. The experiments described here indicate that a similar picture is produced also by varying the degree of sympathetic influence on the organ, by sympathectomy and by spinal depression, the effects of which correspond to those of chemical cystitis and distension respectively. The observation made by Plum and Colfelt (1960) that the spontaneous contractions were augmented by section of the sacral nerves may be explained in terms of an overlooked rise in bladder pressure caused by interruption of sacral afferent impulses which initiate the sympathetic bladder inhibition (Edvardsen 1967).

Discussion

The present experiments indicate that the nervous control of the expulsion phase of bladder activity is brought about by two different mechanisms, one peripheral and one central. The former consists of a sympathetic influence on the bladder pressure while the latter consists of a supraspinal influence on the nervous reflex threshold for micturition. Previous experiments (Edvardsen 1967) indicated that sympathetic inhibition is of spinal origin and essentially independent of supraspinal influence. The present experiments indicate that the tonic supraspinal influence on the micturition reflex is essentially independent of the sympathetic mech-

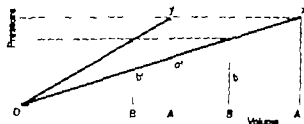


Fig 4 Schematic drawing of the effects shown on the cystometrogram obtained by brain stem transections in intact and sympathectomized cats. OX and OY — bladder tone before and after sympathectomy. OA and OA' — corresponding volumes and a' — corresponding pressure. OB and OB' — volumes after brain stem transection b and b' — corresponding pressure.

The assumption that peripheral sympathetic mechanisms are not involved in the supraspinal tonic influence on the micturition is supported by a further development of the calculations from Table I Thus the effect of sympathectomy has been shown previously to be a steeper rise in the slope depicting bladder tone, while the latter remained unaffected by the various supraspinal transections (Edvardsen 1967a) The present experiments indicate that at steady supraspinal states the micturition reflex is initiated at a critical intravesical pressure, and that this pressure threshold for micturition is not altered by sympathectomy Supposing that the slope of the pressure/volume curve is straight from zero volume up to the micturition threshold in the intact as well as in the sympathectomized animal, the following may be deduced from the schematic cystometrograms shown in Fig 4

In the figure the lines OX and OY represent the pressure/volume curve in the intact and the sympathectomized animal respectively A and A' are the micturition volume thresholds after say supracollicular transection, and a and a' the corresponding intravesical pressures. B and B' are the micturition thresholds after an additional, say intercollicular transection, while b and b' are the corresponding new levels of pressure The lowering of the micturition volume thresholds obtained after intercollicular transection is represented by AB and A'B' in the intact and the sympathectomized animal respectively Since the pressure thresholds are not changed by the sympathectomy $a = a'$ and $b = b'$ In accordance with geometrical laws, $OA/OB = a/b$ and $OA'/OB' = a'/b'$ and thus also $OA/OB = OA'/OB'$ Therefore, if further calculation of the obtained micturition thresholds (Table I) shows no significant fractional differences in the two groups either i.e. that A/B(I) = A/B(II) B/C(I) = B/C(II) and C/D(I) = C/D(II) one may deduce that the effects of the supraspinal transections on the micturition volume threshold are independent of peripheral sympathetic mediation

The results of applying the t test to this problem are seen in Table II using the same symbols as in Table I The p-values indicate that there is no significant difference between the two groups, and thus it may be concluded from this also that there are no grounds for assuming a peripheral sympathetic mediation of the effects of supraspinal transections on the micturition threshold However such participation is not positively excluded.

Ideally the calculations should be based on cystometrograms obtained after the

TABLE II. Calculation of fractional differences in micturition thresholds after brain stem transections in intact (I) and sympathectomized animals (II) (See text.)

	I	x_I	t_I	n_{II}	x_{II}	t_{II}			P
A/B I—II	3	1.80	0.36	3	1.96	1 19	4	0.224	83
B/C I—II	7	0.43	0.29	7	0.38	0.33	12	0.99	40
C/D I—II	8	2.80	1.53	5	3.30	2.11	11	0 46	66

various lesions in the two groups. However as seen in Table I several of the low thresholds were attained after only two, and occasionally one, volume increments, precluding the plotting of the pressure/volume curves.

Little is known about the interplay between the spinal reflex mechanism giving rise to bladder inhibition through the sympathetic nerves and the supraspinal micturition centre giving rise to bladder excitation through the parasympathetic nerves. However it appears from previous experiments carried out by the author (Edvardsen 1967a) and from earlier investigations of the micturition reflex (Sherrington 1892) that the sympathetic as well as the parasympathetic outflow is subserved by afferent impulses mediated through the sacral dorsal roots. Whether these impulses are initiated by the same or by different tension receptors, is not clear Two types of tension receptors have been assumed to exist in the bladder wall, namely one which adapts rapidly and one which adapts slowly on changed tension (Kuru 1965) Any relationship between either of these receptors and efferent fibres has not, however been established.

As regards the supraspinal influence on the two divisions of autonomic bladder nerves, previous transection experiments (Edvardsen 1967a) have not revealed the existence of a tonic supraspinal influence on the sympathetic outflow to the bladder Nor has any tonic parasympathetic influence on the bladder been observed. However stimulating the infundibular midbrain region (Lachtenstern 1912) as well a forebrain, limbic hypothalamic nd mesencephalic structures (Gjone 1965) gave rise to bladder responses mediated through the sympathetic nerves. On the other hand, bladder contraction responses, uninfluenced by sympathetic denervation, ha e been elicited by stim lation of the lateral reticular nucleus and the adjacent grey matter f the lateral reticular formation (Kuru, Kurati and Koyama 1959) nd of the sensori-motor cortical areas I and II the pyriform cortex, the basolateral part of the amygdaloid nuclea complex, and diencephalic and mesencephalic tructures (Gjone 1965) It thus ppears that the sympathetic and the parasympathetic outflow from the cord may both be influenced dependently by upraspinal centres, thereby possibly providing means for the conscious initiation and termination of micturition, irrespective of the actual bladder olume or pressure

References

Bard, P Central nervous mechanisms for the expression of anger in animals. I Reymert, *F eli g nd Emotions*. New York, McGraw-Hill 1950. pp. 11—?37

Barrington, F J F., The effect of division of the hypogastric nerves on frequency of micturition. *Quart. J p Physiol.* 1916 *9* ?61—?64

Barrington, F J F The relation of the hind-brain to micturition. *Brain* 1921 *44* 23—33

Edvardsen P Nervous cont ol of rinary bladder in cats. I. The collecting phase *Acta physiol. sc nd* 1968a. *72* 157—171

Elliott T R., The innervation of the bladder and urethra. *J Physiol.* (Lond.) 1907 *35* 367—445.

Gjone, R., *A Dual Peripheral d S praspin l Autonomic I flue on the Urinary Bladder* Oslo Universitetsforlaget 1965

Gjone, R. nd J Setekleiv Excitatory and inhibitory bladder responses to stimulation of the cerebral cortex in the cat. *Act physiol scand* 1963 *59* 337—348.

Kaada, B. R Brain mechanisms related to aggressive beha ior I *Aggr ssion and Defense* Neural mechanisms and social patterns. Univ Calif Press, Berkeley 1967 (In press)

Kuru M., Nervous control of micturition. *Physiol. Rev* 1965 *45* 425—494

K ru M., T Kurati and Y Koyama, The bulbar esico-constrictor center and the bulbo-sacral connections riding from t. *J omp. Neu ol.* 1959 *113* 365—388

Langworthy O R D L. Ree es nd E. S T cker A tonomic control of the urinary bladder *Brain* 1934 *57* 266—290

Lewin R. J and R. W Porter, Inhibition of spontaneous bladde activity by stimulation of the globus pallidus *Neurology* (Minneap) 1965 *15* 1049—105_

Lichtenstein R., Ueber die zentrale Blaseninnervation, ein Beitrag zur Physiologie des Zwischenhirns *Wien. klin W h* 191 *25* 1248—1249

M rphy J J and H. W Schoenberg, Physiology and surgery of the genitourinary system. *S g Cli N Amer* 1960 *40* 1615—1628.

Plum F nd R H Colfelt The genesis of ental rhythmicity *A ch Neurol.* (Chic.) 1960 487—496

Ruch, T C Central control of the bladder I *H ndbook of Physiology* Baltimore Williams and Wilkin Co 1960 pp 1?07—1?23

Sherrington C S Notes on the rrangement of some motor fibres in the lumbosacral plexus *J Physiol.* (Lond) 1892 *13* 621—772

Stewar C C Mammalia smooth muscle The cat bladder *Ame J Physiol.* 1900. 4 185—208

ang, P C Lev ls of brain stem and diencephalon controlling micturition reflex *J Neurophysiol* 1955 *18* 583—595

ang, P C nd T C Ruch, Non-neurogenic basis of bladder tonus *Amer J Physiol.* 1955 *181* 249—257

Acta physiol. scand. 1968. 72. 183—193

From the Institute of Neurophysiology, University of Oslo, Norway

Nervous Control of Urinary Bladder in Cats

III. Effects of autonomic blocking agents in the intact animal

By

PER EDVARDSEN

Received 31 May 1967

Abstract

EDVARDSEN, P. *Nervous control of urinary bladder in cats. III. Effects of autonomic blocking agents in the intact animal.* Acta physiol. scand. 1968. 72. 183—193.

Bladder motility in anesthetized or decerebrated cats was recorded under isometric conditions before and after the administration of graded doses of various agents with peripheral or ganglionic blocking effects on the autonomic nervous system. Direct or indirect cystometry was employed when the effects on the collecting or on the expulsion phase, respectively, were studied. *Atropine*, which blocks at cholinergic receptor sites, made the micturition contraction inefficient as the ability of the bladder to sustain the initial contraction was abolished. *Hexamethonium*, which blocks ganglionic transmission, impaired the spontaneous micturition contraction as well by also preventing the initial pressure rise from occurring. The drug blocked the sympathetic ganglia at doses smaller than those which blocked the parasympathetic ones. The effects of *propranolol* and *dibenzyline*, which block adrenergic inhibitory and excitatory receptors respectively, suggest that both these receptors are present in the bladder. The former drug increased bladder tone, the latter had an atropine-like effect on the micturition contraction and an excitatory influence on the micturition reflex. Finally, *guanethidine*, which blocks the adrenergic neuro-muscular junction, had an excitatory effect on the bladder and a facilitatory effect on the micturition reflex in addition to its presynaptic blocking effect. These results support previous hypotheses of sympathetic inhibition and absence of parasympathetic activity during the collecting phase, and of bladder pressure as the determinant of the micturition threshold.

In two previous reports on transection experiments (Edvardsen 1967a, b) it was concluded that during the collecting phase of the bladder the intravesical pressure is kept at a low level by a spinal reflex mechanism: afferent bladder impulses mediated through the dorsal sacral roots initiate sympathetic nerve activity which counteracts the rise in bladder pressure on increasing the volume. Further, it was found that the micturition reflex was elicited when the intravesical pressure reached a critical level, and that the decreased volume thresholds of the bladder after sympathectomy was due to a steeper slope of the pressure volume curve — defined as bladder tone (Ruch 1960). Finally, it was concluded that the parasympathetic nerves are activated during micturition only, and that they exert no influence on the bladder during the collecting phase.

Additional support for some of these suggestions might be obtained by the effects of drugs selectively blocking the two components of the autonomic nervous system, and an exploration of this possibility was the purpose of the present study. The drugs selected were atropine, hexamethonium, propranolol, dibenzyline and guanethidine[1]

Material and methods

Thirty adult cats of both sexes weighing 2.5 to 5.1 kg were used. As a general anesthetic pentobarbital sodium (Nembutal® Abbott) was given intraperitoneally in doses of 30 mg/kg, supplemented by intramuscular injections when necessary. Inter- or subcollicularly decerebrated animals were prepared under ether anesthesia, and no recording was attempted until at least one hour had elapsed after transection.

The methods used were largely as described previously (Edvardsen 1967a, b). Direct or indirect cystometry was employed when effects of the drugs on the micturition or the bladder tone, respectively, were assessed. The external jugular or the femoral vein was used for administration of the agents.

The drugs used for investigation were water-soluble except for dibenzyline, which was given in aqueous suspension. Cystometric recording was started 15 min after the administration of atropine, 30 min after hexamethonium, propranolol and guanethidine and 60 min after the administration of dibenzyline.

Results

Eleven out of the 30 preparations employed were decerebrated. Each drug was studied in at least one decerebrated and one anesthetized animal with intact nerve supply to the bladder as well as in one anesthetized animal with extrinsically denervated bladder. The effects obtained in the decerebrated and the anesthetized preparations were essentially similar.

Atropine (6 expts.) in doses of 0.01—0.5 mg/kg body weight was given to the following preparations: one decerebrated and one anesthetized cat with intact nerve supply to the bladder, three anesthetized cats with denervated bladders, and one anesthetized cat with sectioned hypogastric nerves.

The administration of atropine was usually followed by a transient lowering of the blood pressure which amounted to 10 mm Hg, outlasting the period of injection by a few minutes.

In most cases the spontaneous contractions and the bladder pressure were unaffected by the drug. In some animals there was a transient depression, while in others an augmentation was seen. The patterns of bladder motility might also vary with repeated injections in the individual cat. No difference was observed whether the bladder was innervated or denervated.

Repeated cystometric examinations following atropine administration did not reveal any change in the micturition threshold. The micturition contraction on the other hand, changed markedly. This change was characterized by a decreasing ability to maintain the contraction as the doses were increased, whereas the initial pressure

[1] *Hexamethonium* was kindly supplied by May and Baker Ltd., Dagenham, *propranolol* (Inderal®) by Imperial Chemical Industries, Ltd., Manchester and *guanethidine* (Ismelin ®) by CIBA Société Anonyme Basel.

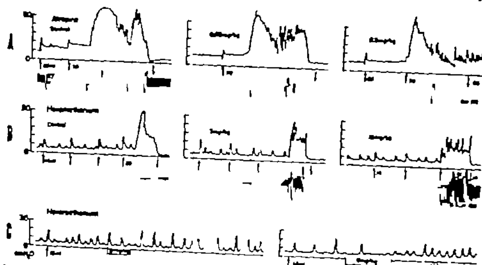


Fig. 1 Effects on micturition (M) of atropine (A) showing the animal' inability maintain the initial contraction, and of hexamethonium (B) showing an over-all pressure reduction. Abdominal straining is reflected in the recording of respiratory excursions. (C) show that 0.10 mg/kg of hexamethonium has no effect, while 6 mg/kg increases bladder motility. Decerebrated cats. Direct cystometry. Volume increments indicated by residual bladder volumes by v

rise was only slightly affected (Fig. 1 A) The residual bladder volume increased correspondingly after attempted oiding. The effect was conspicuous after doses of 0.05 mg kg but was not further increased by doses exceeding 0.2 mg kg

Referring to previous experiments (Edvardsen 1967a, b) th unchanged spontaneous contractions, bladder pressure and micturition threshold after the administration of tropine indicate that the drug does not influence the sympathetic outflow to the bladder Nor does it influence the excitability of the micturition reflex, or block afferent impulses gi ing rise to micturition. The impaired micturition contraction thus suggests that the effects of atropine are confined to the bladder This assumption is supported by stim lation experiments (Edvardsen 1967d) show ng that impulse transmission in the bladder nerves is not blocked by atropine

(b) *H meth nium bromid* (5 expts) was given in doses 0 —60 mg kg to the follow ng preparations one decerebrated and one anesthetized cat with intact nerve supply to the bladder nd three anesthetized cats w th denervated bladders

The injection of large doses of the drug was accompanied by a slight and transient depression of blood pressure, while with doses of less than 15 mg kg no such effects were observed

Doses larger than 0 or 0.4 mg kg tended to augment the pontaneous contractions and to increase the intra esical pressure in cats w th preserved bladder nerves. These effects were also seen with increasing doses (Fig 1 C) and were conspicuous even after 60 mg kg In the denervated bladders on the other hand no change in motility resulted from administration of the drug.

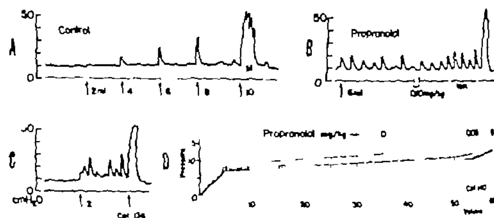


Fig 2. Effect of propranolol on bladder motility Record (A) shows the initial volume threshold for micturition (M) on the direct cystometrogram. Propranolol 0 10 mg/kg produces micturition at a threshold slightly above half the control value shortly after administration (B) ... 30 min later the volume threshold is further reduced (C) The pressure threshold is unchanged in all records. (D) shows the effects of increasing doses of the drug on the pressure/volume curve after spinal cord transection at the level of Th₂. Indirect cystometry Decerebrated cat. Volume increments indicated by ^

The volume threshold for micturition varied inversely with the bladder tone while the pressure threshold was unchanged. With doses of between 1.5 and 3 mg/kg the micturition contraction was slightly altered, showing a decrease in intravesical pressure and a prolonged duration of the contraction. With the aid of abdominal straining, however the bladder was nearly emptied. With larger doses the residual bladder volume increased, and at 12 mg/kg only a few drops of bladder fluid were expelled in spite of forceful abdominal contractions (Fig 1 B)

In view of the results of the transection experiments (Edvardsen 1967a) the decrease in intravesical pressure after small doses of hexamethonium (0.2—0.4 mg/kg) suggests a blockade of the sympathetic ganglia. The alteration of the micturition contraction after 1 5—3 mg/kg on the other hand suggests a blockade of the parasympathetic ganglia as well by these doses. Finally the unchanged pressure threshold for micturition indicates that the drug has no effect on the micturition reflex centre

(c) *Propranolol* (9 expts.) was given in doses of 0.05—6 mg/kg to the following preparations one decerebrated and one anesthetized cat with intact nerve supply to the bladder one anesthetized and three decerebrated animals with the spinal cord sectioned at the level of Th_2, and three anesthetized cats in which the bladder was completely denervated in one and sympathectomized in two.

The injection of propranolol was usually followed by a slight rise in the blood pressure not exceeding 10—15 mm Hg In most cases the pre-injection level was reached within 30 min.

The bladder records from the animals with an intact bladder nerve supply and from those with high thoracic cord transections showed an augmentation of the

pontaneous contractions and a rise in bladder pressure subsequent to the injection, leading to micturition in those preparations with an intact cord (Fig 2 B) Repeated cystometric examinations showed that bladder pressure rose with increasing doses of propranolol up to 1 mg/kg This was seen also after a high thoracic transection of the cord (Fig 2 D) Doses larger than 1 mg kg had no further effect. By contrast, in the sympathectomized preparation and in the cat with a denervated bladder the drug caused no significant effect on bladder motility

The volume threshold for micturition varied inversely with the bladder tone while the pressure threshold remained constant, i.e after any dose of propranolol the micturition reflex was elicited at lowered volumes but at the same bladder pressure, namely the control value (Fig. 2 C) Micturition emptied the bladder completely after any of the doses of propranolol. Neither the magnitude nor th duration of the contraction were influenced by the drug. In particular no observation suggested any hindrance at the bladder outlet.

The lack of effect in sympathectomized and denervated bladders, in contrast to the marked effect in those which are fully innervated, indicates that the effect of propranolol is related to the influence exercised by the hypogastric nerves. Moreover the effect on the bladder suggests a release of inhibition. The persistence of this effect after high thoracic cord transections supports the assumption of a spinal origin of this inhibition (Edvardsen 1967a)

(d) *Dibenzyline* (4 expts.) was given in doses of 0.01—1 mg kg to the following preparations two decerebrated cats with ntact nerve supply to the bladder one anesthetized cat with spinal cord sectioned at the level of Th and one anesthetized cat with denervated bladder No effect on the blood pressure was observed.

The spontaneous bladder contractions and the intravesical pressure remained un hanged for t least 1 hr after injection of the drug. Similarly the bladder tone was seen on repeated cystometrography to be unaltered by dibenzylamine in either of the preparations. In particular direct cystometry did not reveal any signs of incontinence.

The volume threshold for micturition was, however gradually lowered as the dose was increased, the pressure level at which the reflex was elicited being reduced (Fig. 3 A, B, and C) These effects were observed after doses of 0 01—0 5 mg kg of the drug and no further effect was obtained after 1 mg kg. The micturition contraction was also affected, becoming less effective with ncreasing doses In one of the decerebrated animals a dose of 0.1 mg kg reduced the voiding act to a single jet which expelled few millilitres of fl id only while in the other the same effect was observed after doses exceeding 0.5 mg kg (Fig 3 C)

On the basis of the results of previous experiments (Edvardsen 1967a) the lack of effect on intra esical pressure indicates that adrenergic motor receptors do not oper te during the collecting phase of bladder activity On the other hand, the decreased pressure threshold for micturition suggests the operation of n excitatory effect on the micturition reflex (Edvardsen 1967b) The reason for the tropine-like effect on the micturition contraction is not clear

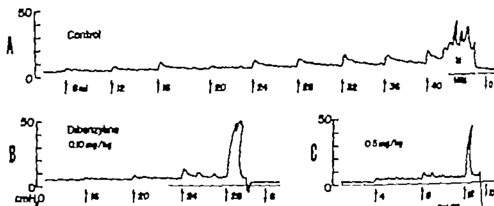


Fig 3 Effect of dibenzyline on bladder motility The pressure threshold for micturition (M) as determined initially (A) is reduced by 0.1 mg/kg (B) and still further by 0.5 mg/kg (C) At the same time the contraction is rendered inefficient. The slope of the pressure/volume curve is unchanged Decerebrated cat. Direct cystometry Volume increments indicated by ↑ residual bladder volumes by ↓

(c) *Guanethidine* (6 expts.) was given in doses of 0 1—8 mg/kg to the following preparations one decerebrated and two anesthetized cats with intact bladder nerves, two decerebrated cats with additional spinal cord transection at the level of Th_5 and one anesthetized cat with an extrinsically denervated bladder

On injection of the drug there was a rapid rise in blood pressure of some 10—30 Hg depending on the dose, followed by a gradual fall to normal or slightly de-ved values within 10—15 min.

The immediate effect on the bladder was a decrease in pressure and a reduction in the amplitude and frequency of the spontaneous contractions that lasted for the period of blood pressure elevation. Subsequent to this initial depression there was a gradual augmentation of the spontaneous contractions to above the control level, and a rise of the bladder pressure, sometimes leading to micturition (Fig 4 B) Definite effects occurred after 0 1 mg kg and were usually at maximum after 0.5 mg kg However with respect to the spontaneous contractions and the pressure the denervated bladder behaved qualitatively similarly to the bladders of the intact and the spinal animals (Fig 4 D)

The volume threshold for micturition was reduced by guanethidine. This reduction was only in part due to the steeper pressure volume curve of the bladder the other cause being a decreased pressure threshold, i.e after guanethidine the micturition reflex was elicited at pressures lower than those giving rise to bladder emptying in the control. The micturition contraction, on the other hand, was not influenced, and complete bladder emptying occurred after all doses used (Fig 4 C)

Although in accordance with the results of previous transection experiments (Edvardsen 1967a, b) the additional effect on the denervated bladder suggest various modes of action of guanethidine on this organ.

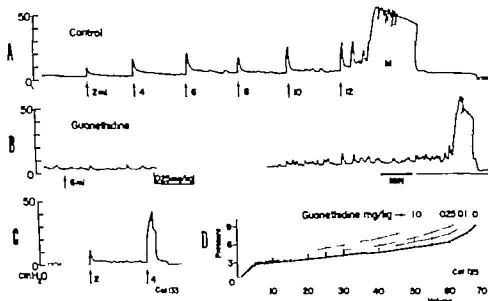


Fig 4 Effect of guanethidine on bladder motility The initial thresholds for micturition (M) determined at (A) Guanethidine 0 25 mg/kg, at half the volume threshold causes transient depression followed by rise in pressure and micturition at slightly reduced pressure threshold (B) while 30 min later the threshold is further reduced (C) (D) show the effect of increasing doses on the pressure/volume curv after spinal cord transection t the lev l of Th5. Indirect cystometry Decerebrated cats. Volume increments indicated by ^

(f) *Int aspinal proc dur after guanethidi e d propranolol* (3 expts.) It has been shown previously (Edvardsen 1967a) that the depression of bladder motility observed after intraspinal lesions is due to a sympathetic discharge evoked by the procedure, nd that such non-specific bladder depression does not occur if the hypogastric nerves are sectioned beforehand. The present experiments aimed at determining whether the bladder response to such a procedure was changed by agents blocking the sympathetic outflow or the inhibitory receptor sites.

In one subcollicularly decerebrated cat the spinal cord was transected t the level of L_1 after the administration of 5 mg kg of guanethidine (Fig. 5 A) Apart from two peaks of intravesical pressure due to twitches nduced in the animal by respectively ligation and transection of the cord, no changes in the spontaneous contractions or the bladder pressure occurred.

In one decerebrated and one anesthetized animal the cord was transected at the levels of L_1 and L_1 after the administration of 0 1 nd 6 mg kg of propranolol respectively In none of the preparations did any sign of bladder depression occur More over the cat receiving the larger dose showed prolonged contraction response of the bladder lasting for several minutes (Fig. 5 B) This period corresponded to that of the maximum bladder depression seen when th cord of an untreated animal is transected. The pressure raising effect of propranolol by the ncreased sympathetic

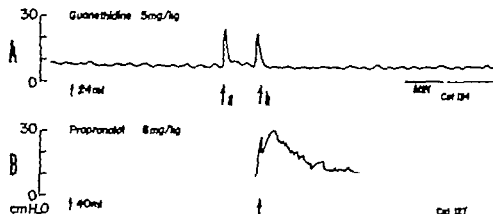


Fig 3. Effects of guanethidine (A) and propranolol (B) on the bladder response to spinal cord transection (large arrows). Pressure elevations at *a* and *b* are due to twitching of the animal on ligation and transection of the cord. Cat 134 decerebrated subcollicularly, cat 127 anesthetized. Indirect cystometry. Bladder volumes indicated by ↑.

discharge due to cord transections has been further substantiated in stimulation experiments (Edvardsen 1967d).

These results thus support the hypothesis that the bladder depression following intraspinal procedures is due to sympathomimetic inhibition and not to removal of any parasympathetic excitatory influence. Furthermore, the prolonged contraction of the bladder in response to the increased sympathetic discharge after the administration of propranolol suggests the presence of adrenergic excitatory receptors in the bladder in addition to the inhibitory receptors responsible for the bladder depression observed in the untreated animal.

Discussion

The present experiments show that bladder motility is changed significantly by drugs known to interfere with impulse transmission in the autonomic nervous system. The effect of atropine is to impair the micturition contraction such that the initial level of pressure cannot be maintained, most probably because the cholinergic receptors on the smooth muscle cells are blocked. Due to blockade of the parasympathetic ganglia the micturition contraction is also abolished by hexamethonium, preventing the initial pressure rise as well. However, the drug appears to block the sympathetic ganglia when given in doses smaller than those causing parasympathetic ganglion blockade, as indicated by a steeper pressure volume curve. A steeper slope of this curve is also obtained after the administration of propranolol and guanethidine, which are known to block adrenergic inhibitory receptors and adrenergic neuro-effector junctions respectively. However, the last mentioned drug also has an excitatory effect on the denervated bladder and on micturition. Finally dibenzyline, which blocks adrenergic excitatory receptors, does not influence bladder tone. This drug, however, has an atropine-like effect on the micturition contraction and lowers the nervous reflex threshold for micturition.

These results are in accordance with the previous assumptions (Edvardsen 1967a,

b) that the parasympathetic nerves mediate impulses for the micturition contraction only while the sympathetic impulses inhibit bladder tone during the collecting phase and thus raise the volume threshold for micturition. The persistence of the effects after section of the upper thoracic portion of the spinal cord supports the assumption of a spinal origin of the sympathetic inhibition. Likewise the earlier suggestion of an excessive sympathetic discharge, caused by various intraspinal procedures, as the mechanism responsible for the bladder depression, is supported by these experiments, since this effect was abolished by guanethidine and propranolol. However in the last-mentioned case the sympathetic discharge provoked a prolonged bladder contraction which may indicate the presence of adrenergic excitatory receptors in this organ.

The cholinergic transmission mechanism in bladder parasympathetic responses has been elucidated in several recent investigations (Guyernek 1961 Garrett 1963 Vanov 1965 Hcović, Rand and Vanov 1965 and others) Concerning atropine, the blocking effect of this drug on the bladder is known to be weak (Ursillo 1961) In the present experiments the effects of atropine on micturition appear to be similar to those reported by Henderson and Roepke (1935) who found that this drug abolished the bladder response to pelvic nerve stimulation after the initial contraction. Ursillo and Clark (1956) did not observe this effect at stimulation frequencies of 15 cps. Employing different stimulation frequencies (Edvardsen 1967d) it was found that at 30 cps atropine abolished the sustained latter part of the bladder contraction, while leaving the initial part unaltered, while at 5 cps the sustained contraction was preserved. From the effect of atropine in these experiments it may be postulated that th pelvic nerves fire at frequencies of 15—30 cps during spontaneous micturition contraction.

The suggested sympathetic ganglion blockade by hexamethonium in doses smaller than those which block the parasympathetic ganglia is in accordance with similar observations made on dogs by Garrett (1963) Nesbit *et al.* (1947) blocked the micturition contraction in man with hexamethonium, but did not report any effects on the bladder pressure. In the present experiments a dose 20-times larger than that which blocked the micturition contraction did not reduce the bladder pressure or the spontaneous contractions. On the contrary the spontaneous bladder motility increased with increasing doses, probably due to a progressive blockade of sympathetic ganglia. These observations lend strong support to the assumption that the parasympathetic nerves have no excitatory influence on the bladder during its collecting phase (Edvardsen 1967a) Similar conclusions were reached by Plum (1960) and Plum and Colfelt (1960) who were unable to abolish spontaneous bladder contractions by the use of tetraethyl-ammonium chloride.

The pressure-raising effect of propranolol may reasonably be attributed to blockade of adrenergic inhibitory receptors present in the same or in cells others than those containing the excitatory receptors (Burnstock and Holman 1966) On the other hand, the unchanged micturition contraction after the administration of this drug suggests that adrenergic inhibitory receptors do not operate during the expulsion

of urine and, in particular that no mechanism involving these receptors is required to allow the bladder outlet to open. The lack of effect on denervated and sympathectomized bladder seems to indicate that the sympathetic influence on bladder motility is mediated mainly through the hypogastric nerves, as was also suggested to be the case in the dog (Garrett 1963) Central depressant effects have been reported by Murman, Almirante and Saccani-Guelfi (1966) and in the present experiments respiratory failure proved fatal on several occasions in attempted experiments on decerebrated preparations. In the preserved preparations, however the pressure threshold for micturition was not altered by propranolol, indicating a non-influence on the nervous reflex threshold

Dibenzyline did not affect the bladder pressure significantly which indicates that adrenergic excitatory receptors are not brought into play by the collection of urine The continence of the bladder on the other hand suggests that the elastic forces at the bladder outlet are capable of keeping the latter closed without muscular support induced by adrenergic excitatory receptors. This was also suggested by Bro-Rasmussen *et al.* (1965) from anatomical and functional investigations. The excitatory influence of dibenzyline on the micturition reflex is consistent with the convulsant effect on the central nervous system produced by 2 halogenoethylamines. The atropine like effect on the micturition contraction may be due to the affinity of this type of adrenergic excitatory receptor blockers for cholinergic receptor sites as well (Triggle 1965) The possibility of a blockade of adrenergic excitatory receptors operating during micturition by dibenzyline is, however not excluded by these experiments.

The blood pressure elevation and the depression of bladder motility that followed immediately upon the injection of guanethidine are probably due to the well known initial release of catecholamines (Muscholl 1965) The subsequent enhanced motility may however be due to various properties of the drug including presynaptic blockade of the release of the transmitter potentiation of responses of effectors to noradrenaline, and prolonged release of transmitter also in the denervated bladder (for ref see Triggle 1965) as well as to an excitatory effect on the micturition reflex Although compatible with the previous suggestions as to nervous influences acting on the bladder (Edvardsen 1967a, b) the results of the guanethidine experiments afford little additional information.

The functional significance of the sympathetic bladder innervation is not clear From previous and recent experiments (Barrington 1916, Gjone 1965 Edvardsen 1967b) it is known that an apparently normal micturition contraction also takes place in the absence of sympathetic nerves, although it occurs at a decreased volume Similarly in the present experiments guanethidine did not cause any alteration of the contraction. According to these experiments adrenergic inhibitory receptors operate only during the collecting phase of the bladder and not during micturition. Adrenergic excitatory receptors, on the other hand do not seem to influence the pressure volume curve while a participation of these receptors in the micturition contraction is not definitely excluded. Even if doubt thus arises whether adrenergic excitatory

receptors exist in the bladder their presence is suggested by the prolonged bladder contraction produced by intraspinal procedures carried out after the administration of propranolol. Normally such procedures cause a marked relaxation of the bladder due to increased sympathetic discharge (Edvardsen 1967a) In the present experiments the depression was blocked by guanethidine and converted to contraction by propranolol. It may be assumed, therefore that both excitatory and inhibitory adrenergic receptors are present in the bladder as in the uterus and other viscera (for ref., see Nickerson 1965)

References

BARRINGTON, F J F The effect of division of the hypogastric nerves on frequency of micturition *Quart. J exp Physiol.* 1916. *9* 261—264

BRO-RASMUSSEN, F A. HALBORG SØRENSEN, E. BREDAHL and A. KELSTRUP The structure and function of the urinary bladder *Urol. int* (Basel) 1965. *19* 280—295

BURNSTOCK, G and M. E. HOLMAN Effects of drugs on smooth muscle. *An Rev Pharmacol* 1966. *6*. 129—156.

EDVARDSEN P Nervous control of urinary bladder in cats. I. The collecting phase. *A t physiol. scand* 1968a. *72* 157—171

EDVARDSEN, P Nervous control of urinary bladder in cats II. The expulsion phase. *Acta physiol. scand.* 1968b. *72* 172—182.

EDVARDSEN P Nervous control of urinary bladder in cats. IV Effects of autonomic blocking agents on responses to peripheral nerv stimulation *Act physiol. scand* 1968d. *72* 234—247

GARRETT J. Selective blockade of sympathetic or parasympathetic ganglia. *Ar h. int Pharmacodyn.* 1963. *144* 381—391.

GJONE, R., *A Dual Peripheral and S praspinal Aut omic Influe on the Urinary Bladder* Oslo Universitetsforlaget 1965.

GYERMEK, L., Cholinergic stimulation and blockade of urinary bladder *Amer J Physiol.* 1961 *201* 325—328.

HENDERSON V E. and M. H. ROEPKE, The urinary bladder mechanisms *J Pharmacol. exp Ther* 1935. *54* 408—414

HUKOVIĆ, S M. J RAND and S. VANOV Observations on an isolated, innervated preparation of rat urinary bladder *Brit. J Pharmacol.* 1965 *24* 178—188

MURMANN, W L. ALMIRANTE and M. SACCANI-GUELFI. Central nervous system effects of four β-adrenergic receptor blocking agents. *J Pharmacol* 1966. *18* 317—318

MITCHELL, E., Drugs interfering with the storage and release of adrenergic transmitters. In. Koelle, *Pharmacology of Cholinergic and Ad energic T ansmission* New York, Pergamon Press 1965 pp. 291—302.

NESBIT R. M., J LAPIDES, W W VALK, M SUTLER R L BERRY N H LYONS, K N CAMPBELL and O. K. MOE, The effects of blockade of the autonomic ganglia of the urinary bladder in man. *J U ol* (Baltimore) 1947 *57* 242—.30

NICKERSON M Adrenergic receptor mechanisms In Koelle *Pharmacology of Cholinergic and Ad energic T ansmission* New York, Pergamon Press 1965 pp 303—314

PLUM, F Autonomous urinary bladder activity in normal man. *A ch Neurol* (Chic) 1960 *2* 497—503

PLUM F and R. H COLFELT The genesis of vesical rhythmicity *A ch Neu ol* (Chic) 1960 *2* 487—496

RUCH, T C Central control of the bladder In *Handbook of Phy iology* Baltimore Williams and Wilkins Co 1960 pp. 1207—1223

TRIGGLE, D J *Chemical A pect of the A tonomic Nerv us System* London and New York, Academic Press 1965 pp 329

URSILLO R C., Investigation of certain aspect of atropine-resistent nerve effects. *J Pharmacol. exp Ther* 1961 *131* 231—236

URSILLO, R C. and B B CLARK, The action of tropine on the urinary bladder of the dog and on the isolated nerve—bladder strip preparation of the rabbit *J Pharm ol p Ther* 1956 *118* 338—347

VANOV S., Responses of the rat urinary bladder *in sit* to drugs and to nerv stimulation. *B it J Pharmacol* 1965 *24* 591—600

Acta physiol. scand. 1968. 72. 194—199

From the Division of Clinical Pharmacology Department of Pediatrics, Karolinska Sjukhuset, Stockholm, Sweden

Demonstration of a Receptor Reserve for Acetylcholine in the Human Fetus

By

LARS O. BORÉUS

Received 15 June 1967

Abstract

BORÉUS, L. O. *Demonstration of a receptor reserve for acetylcholine in the human fetus.* Acta physiol. scand. 1968. 72. 194—199.

The development of the acetylcholine (ACh) receptor function in man was studied by means of determination of dose-effect curves for ACh action upon fetal smooth muscle tension in vitro. Segments of ileum from human fetuses of various gestational ages (6.5—20.5 cm head-rump length) were used. Phenoxybenzamine (POB) blocked irreversibly the spasmogenic action of ACh. Exposure of the tissue to low concentrations of POB for a short period led to a shift of the subsequent dose-effect curve for ACh along the dose-axis. By varying the length of the POB exposure period (4—10 min) different degrees of shift, i.e. different degrees of antagonism could be produced. It was found that the shift could amount to as much as about one logarithmic unit with preserved maximum of the dose-effect curves. This means that activation of only a fraction of ACh receptors is enough for maximal response. Thus, the phenomenon of a receptor reserve to ACh was demonstrated. Furthermore the extent of this reserve was not significantly changing during the fetal age period studied. It is concluded that the characteristics of ACh receptor function in man are fully developed in early fetal life.

An important aspect of drug action is the quantitative relation between the concentration of agonist in the vicinity of the receptors and the resulting effect. This relation can be studied by means of determination of the dose-effect curve for a drug in a simplified biological system. In a previous paper (Boréus 1967) this curve was used to characterize the development of acetylcholine receptor function during ontogenesis in man. It was found that the dose of acetylcholine needed to produce a given proportion of the maximal effect (isometric tension in segments of ileum from human fetuses) did not change with fetal growth during the middle trimester in spite of the fact that the absolute maximum response greatly increased with the development of the fetus. Also the concentrations of atropine and pethidine (meperidine) for a given degree of antagonism were constant during the same fetal age period.

In the present paper the development of the human acetylcholine receptor function was studied from another point of view. It has been shown in animal experiments that there might exist a "surplus" of receptors, i.e. that many more receptors are present than are required for maximal response (Stephenson 1956, Nickerson 1956). In this study the possible existence of such a "receptor reserve" in fetal tissues was investigated by means of determination of the dose-effect curve before and after partial inhibition with small doses of phenoxybenzamine. This drug produced an irreversible blockade of the acetylcholine effect. A parallel shift of the curve along the dose-axis without loss of *maximum* effect was taken as proof of the existence of a receptor reserve. It was found that this phenomenon could be demonstrated in all fetal ages studied.

Methods

Human fetuses were obtained at therapeutic abortions and immediately brought to the laboratory. Four consecutive segments of ileum were each suspended in jacket-warmed 20 ml baths with Tyrode solution at pH 7.4 and 37° C. The isometric tension produced by addition of acetylcholine to the bath was recorded by means of strain gauge transducers and a polygraph. The technical details have been described earlier (Boréus 1967).

When stable responses to repeated doses of acetylcholine were obtained a cumulative dose-effect curve was determined by adding increasing concentrations of acetylcholine without washing between the administrations. When a further increase in response could not be produced by a further increase in dose, the fluid was changed and the segment allowed to relax to its initial resting tension. A second dose-effect curve was then produced in the same way and used in the comparisons with the subsequent curves in which the segment had been treated with inhibitor. The second curve was chosen as standard for comparison since the first one usually had a lower maximum (see Table I).

After determination of the second control dose-effect curve in this way a dose of the inhibitor phenoxybenzamine (POB) was added to the fluid in two of the four baths and allowed to interact with the ileum segments for a measured time period, usually 4 to 10 min. After this exposure which in itself did not produce any change in the resting tension, the inhibitor was washed out of the bath. A resting period of 10—15 min was then allowed to pass during which two or three additional washings were made. It was found that essentially all of the reversible part of the POB blockade had disappeared during this period and that the remaining antagonism was very stable. A new cumulative dose-effect curve was now determined for comparison with the second control curve for the same segment. If the same maximal response as in the second control curve could be obtained, a new dose of POB was added and allowed to block another part of the population of acetylcholine receptors. After washing and allowing a resting period as before a new cumulative dose-effect curve was determined. In this way periods of exposure to POB alternated with dose-effect curves until the maximum response in the second control curve could no longer be obtained. The shift along the dose-axis between the second control curve and the last curve with which the full maximum could be reached was taken as a measure of receptor reserve to acetylcholine. Two of the baths were not treated with inhibitor between the subsequent dose-effect curves. These two segments thus served as controls so that any signs of fatigue could be detected immediately.

Results

The demonstration of the existence of a receptor reserve phenomenon by means of graded irreversible inhibition involves comparisons of repeated dose-effect curves in the biological system used. These curves must be reproducible both in terms of maximum and slope during a time period long enough to permit the entire experiment.

TABLE I Maximal isometric tension in human fetal ileum segments following repeated exposure to acetylcholine. Values obtained from 6 consecutive dose-effect curves in 19 segments from fetuses of 6.5—20.5 cm head to rump length. Mean values and standard error given in per cent of individual differences from the second control curve

Curve no.	n	Mean difference ± S.E. from value obtained in curve no. 2 per cent	Statistical significance
1	19	-11.1 ± 2.4	$p < 0.001$
	19	0	
3	19	$+2.8 \pm 1.7$	$p > 0.05$
4	19	$+0.4 \pm 2.6$	$p > 0.05$
5	19	-3.4 ± 2.0	$p > 0.05$
6	19	-6.5 ± 3.6	$p > 0.05$

Stability of maximum and slope of the dose-effect curve The acetylcholine dose-effect curve is remarkably stable in the ileum of human fetuses of all gestational ages studied. However as described earlier (Boréus 1967) the first curve obtained usually has a somewhat lower maximum than the subsequent, more uniform curves in the same preparation. The variation of maximum response in 19 ileum segments from 3 different human fetuses (range 6.5—20.5 cm head-rump length) is shown in Table I It is seen that the maximum of the first control curve is significantly lower than that from the second control curve but that the subsequent control curves do not significantly differ in this respect from the second curve although there is a slight tendency towards decline. Furthermore 4 to 6 control dose-effect curves could usually be produced without any significant change of the slope of the curves. Thus, the preparation had the required stability for a period of sufficient length so that a complete experiment could be carried out involving two consecutive control curves without inhibitor followed by a series of three or four "experimental" curves, each preceded by a period of exposure to POB. The maxima and slopes of these POB-inhibited curves were compared to the second control curve

There was no difference in stability of the control curves in the various ages of the fetuses.

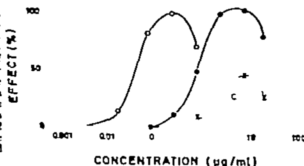
Effects of exposure to POB The response to acetylcholine was, in all cases, inhibited by previous exposure to POB. The blockade developed within a few minutes and was reasonably stable after about 15 min. It was found that 0.2 μg/ml of POB was a suitable standard concentration for producing a blockade of the response to acetylcholine. The degree of block was depending upon the length of time of exposure to the inhibitor In most cases, a time period of 4 to 6 min, followed by wash-out of the antagonist, was found to produce a shift of the subsequent dose-effect curve along the dose axis without decrease of the maximum Arbitrarily a decrease of maxi-

TABLE II. Demonstration of acetylcholine receptor reserve in ileum from human fetuses of various gestational ages

Size of fetus (head-foot/head-rump length) cm	Time of exposure to 0.2 ug/ml POB min	Change of maximum isometric tension in per cent of second control curve	Shift of the dose effect curv (log units)
30.0/20.5	6	+7	0.75
	6+6	—2	1.0
29.0/20.0	4	+2	0.75
	4+4	—2	0.75
	4+4+4	—4	1.0
28.0/20.0	5	—2	0.5
25.0/18.0	4	—4	1.25
25.0/17.0	6	—5	0.5
24.0/16.5	2	—5	1.0
23.0/16.0	8	—5	0.5
20.5/14.5	6	—4	0.75
	6+4	—4	1.0
18.0/13.0	4	—1	0.25
	4+4	—4	0.25
	4+4	—5	0.5
12.5/9.0	10	2	0.25
11.0/8.0	4+8	+9	0.5
9.0/6.5	10	+5	1.0

I 5 expts. (fetal head-rump length 16.5, 16.5, 13.0 15.0, and 13.0 cm) the shift of the curv along the dose axis following treatment with POB was associated with depression of the maximum of more than 5 per cent. These experiments re not included in the table.

Fig 1 Demonstration of ACh receptor reserv in ileum from human fetus (6.5 cm HR-length) Concentration of ACh given as base A the second control dose-effect curv (without previous exposure to inhibitor) B dose-effect curv following exposure of the tissue to POB (0.. µg/ml for 10 min) Note the shif of the curv along the dose-axis with preserved maximum value C: dose-effec curve following an additional exposure to POB (0.2 µg/ml for 10 min) Note that the shif of the curv is now associated with depression of maximum.



mum was defined as a value which was more than 5 per cent lower than the value of the second control curve.

Using these criteria a receptor reserve was demonstrated in all fetal ages studied. There was a certain degree of individual variation in sensitivity to POB, and in some specimens a second and sometimes even a third period of exposure to POB could further shift the curve without loss of maximum. On the other hand in the case where the sensitivity of POB in the ileum was high, the standard treatment with 0.1 μg ml for 4 min was too strong so that a shift of the dose-effect curve for acetylcholine was associated with depression of the maximum i.e the spare receptors had all been blocked and the receptor reserve could therefore not be demonstrated. In all these cases, however the depression of the maximum was associated with a shift of the curve along the dose axis. A decrease of the maximum without a shift of the curve was not seen.

The results of all the POB experiments are summarized in Table II and the appearance of the sets of dose-effect curves from a complete experiment is shown in Fig 1

Discussion

One of the basic assumptions made by Clark (1937) in elaborating the laws for drug receptor interaction was that the percentage of receptors occupied is equal to the percentage response of the tissue. Hence when 50 per cent of the receptors are occupied there is a 50 *per cent response possible, and when the response is maximal* all the receptors are occupied. Clark himself pointed out that this assumption might be incorrect. In fact, it has been shown by two basic methods that the response obtained is not linearly related or proportional to the number of receptors occupied (1) The use of partial agonists (Stephenson 1956) and (2) The use of "non-equilibrium blockade (Nickerson 1956) The results with both methods suggest the existence of spare receptors For instance the latter author showed that only 1 per cent of the receptors are necessary to produce a maximum response to histamine in guinea pig ileum In terms of the rate theory of drug action (Paton 1961) "spare *receptors" are equal to spare capacity for association of the drug molecules* to the receptors.

The phenomenon of receptor reserve constitutes an intimate property of pharmacological receptors in the tissues. Therefore it was of interest to analyze if it all existed in fetal tissues and, if so, whether it was in some way changing during maturation of the tissue. The experiments in this study show clearly that acetylcholine "spare" receptors exist in ileum even in small human fetuses (Fig. 1) It is also evident that the degree of shift of the dose-effect curve, i e the proportion of receptors that are in excess is not changing during the development in spite of the fact that the absolute maximum effect is *continuously increasing with age* of the fetus.

Thus, the present experiments are in agreement with the earlier finding (Boréus

1967) that the acetylcholine receptor function is well developed early in human fetal life and that its relationship to the specific agonist is not quantitatively changing during ontogenesis.

This work was supported by grants from the Swedish Medical Research Council (Project No. 14X 522), Läkemedelsindustriföreningens fond för klinisk farmakologisk forskning and Prenatalforskningsnämnden. The author is indebted to Mrs. Siv Leander for excellent technical assistance.

References

Boréus, L. O. Pharmacology of the human fetus. Dose-effect relationships for acetylcholine during ontogenesis. *Biol. Neonat.* (Basel) 1967 *11* 328—337

Clark, A. J. I. Heffter *Handbuch der experimentelle Pharmakologie. Ergänzungswerk.* Berlin, Springer 1937 Bd. 4

Nickerson, M., Receptor occupancy and tissue response. *Nature* (Lond.) 1956. *178*. 697—698.

Paton, W. D. M., A theory of drug action based on the rate of drug-receptor combination. *Proc. roy. Soc. B* 1961 *154* 1—69

Stephenson, R. P. A modification of receptor theory. *Brit. J. Pharmacol.* 1956 *11* 379—393

Acta physiol. scand. 1968. 72. 200—204

From the Department of Physiology, University of Göteborg, Sweden

Tonic Supraspinal Mechanisms Influencing the Intestino-Intestinal Inhibitory Reflex

By

BÖRJE JOHANSSON, OLOF JONSSON and BENGT LJUNG

Received 15 June 1967

Abstract

JOHANSSON, B., O. JONSSON and B. LJUNG. *Tonic supraspinal mechanisms influencing the intestino-intestinal inhibitory reflex.* Acta physiol. scand. 1968. 72. 200—204.

Supraspinal control mechanisms acting on the spinal intestino-intestinal inhibitory reflex have been studied with regard to their possible tonic influence. The experiments which were done on chloralose or chloralose-urethane anesthetized, vagotomized cats involved acute experimental lesions at the mesencephalic and cervical spinal levels of the CNS. The results indicate that the autonomic reflex is subjected to facilitation from suprabulbar areas and to strong tonic suppression from bulbar structures.

Supraspinal control systems have been described for several different propriospinal reflexes. They have been studied mainly by topical electrical stimulation of the central structures or by experimental lesions in the CNS. The latter type of experiments has indicated a *tonic* influence from higher centres on the spinal reflexes. Thus brain stem structures have been demonstrated to exert a tonic inhibitory effect on skeletal muscle reflexes initiated from both somatic and visceral afferents (Downman and McSwiney 1946, Holmqvist and Lundberg 1959, Yamamoto and Araki 1963). Wang and Brown (1956a, b) and Wang, Stein and Brown (1956a, b) showed that such a tonic restraint affects also the autonomic galvanic skin reflex.

We have reported in a previous article (Johansson, Jonsson and Ljung 1965) that electrical stimulation of certain bulbar structures inhibits the intestino-intestinal inhibitory reflex in anesthetized cats. The aim of the present investigation was to determine whether these structures would also display a tonic influence on this autonomic gastro-intestinal reflex.

Methods

Fifteen experiments were carried out on cats under chloralose (50 mg/kg) or chloralose — urethane anaesthesia (40 and 80 mg/kg respectively) The operative procedures have previously been described in detail (Johansson, Jonsson and Ljung 1965) I principle tw separat loops of intestine with intact blood and nervous supply were prepared from the proximal jejunum. The motility of one loop was recorded as volume changes by means of piston recorder system. The other loop was used for eliciting the intestino-intestinal inhibitory reflex by graded distensions. The vagi were cut bilaterally and the adrenals tied off after corticoid substitution had been given by intramuscular hydrocortison (2 mg/kg) A hole was drilled in the occipital bone and the central part of the cerebellum was sucked out to expose the brain stem where transections were to be made The animals were artificially entilated.

After series of control responses had been induced by graded intestinal distensions, partial or complete transections were done at the intercollicular level. The effects of these experimental lesions on the intestino-intestinal reflex were studied by repeating the series of distensions. This was done also after the influence of the lower bulbar regions had been excluded by sectioning the spinal cord t the upper cervical level. Infiltration of these cord segments with 2 % lidocaine or local cooling with ethyl-chloride prevented the acute intense sympathetic discharge which would otherwise appear as the cord was dissected part.

After the experiments the brains were fixed in 10 % formaline solution and sectioned to identify the localizations of the lesions.

Results

Fig 1 illustrates recordings of arterial blood pressure and motility in the proximal jejunum from a representative experiment where the effects of lesions at different levels of the CNS on the intestinal inhibitory reflex were studied. Th upper record shows a series of control responses elicited by intestinal distension to different pressure levels. W'th the brain stem intact, promt inh'bition of intestinal tone and motility was obtained by distension pressures of 20 cm H_2O or more in this animal. After an extensive medial brain stem lesion had been made at the intercollicular level (A in Fig 2) the minimum pressure required to give intestinal inhibition increased from 20 to approximately 50 cm H_2O as is seen in the middle record of Fig 1 Complete spinal cord transection at a cervical level (B in Fig 2) resulted in a pronounced decrease of intestinal tone and motility and a decreased threshold for eliciting the intestinal inhibitory reflex so that it again appeared t distension pressures of 30 and 20 cm H_2O (left panel of lower record in Fig 1) The low intestinal tone after spinalization is not due to impaired circulation in connection with the f ll in blood pressure but to neurogenic inhibition as shown by the result of infiltration with 2 % lidocaine around the periarterial nerves of the superior mesenteric artery (arrow in Fig 1) Reflex inhibition of intestinal tone was not obtained by distension fter this procedure.

An increase in the threshold distension pressure required for eliciting reflex inhibition of intestinal motility was seen after subtotal or complet mesencephalic transections in 1 of the 15 expts. The level of intestinal tone nd the amplitude of the phasic contractions prevailing between the distension periods were often increased fter these lesions in comparison with the control tuation Transection of the cervical spinal cord invariably led to a decrease in intestinal tone often down to level comparable to that reached at strong reflex inhibition by supramaxim

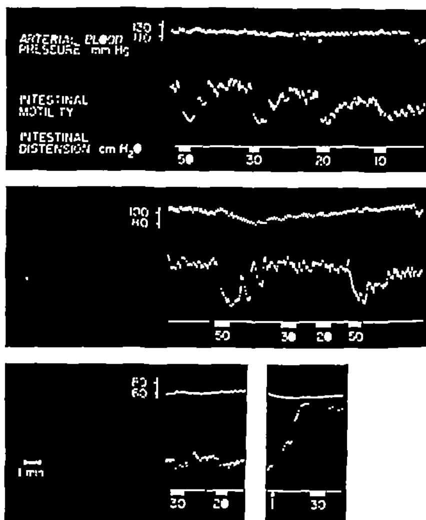


Fig 1 Cat 2.7 kg, chloralose Effects of experimental central nervous lesions on intestinal motility and on the reflex responses to graded gut distensions
Top recording Control period. Prompt intestinal inhibition is obtained by distending another loop of intestine with pressures of 20 cm H_2O or more.
Middle recording After lesion at the intercollicular level (A, Fig 2) Distension pressures of 50 cm H_2O are required to elicit intestinal inhibition.
Lower recording Left panel After cervical spinalization the level of intestinal tone is decreased and reflex inhibition can again be elicited by 20 cm H_2O distension. Right panel Effects of extrinsic denervation of the gut The periarterial nerves supplying the intestine are infiltrated with lidocaine (at arrow) Note the marked increase in intestinal activity and the absence of response to gut distension.

tending pressures. In spinal animals where some basal intestinal tone still persisted, reflex inhibition was elicited by distending pressures lower than those required before the cord transection (e.g. Fig 1) Extrinsic denervation of the gut by spinal anesthesia or by infiltration of the mesenteric root with a local anesthetic agent consistently increased intestinal tone to a very marked degree in the spinal animals.



Fig. 2. Horizontal section of cat' brain from the experiment illustrated in Fig. 1
A. Medial lesion t the collicular level.
B. Complete transection of the spinal cord.

Discussion

The inhibition of intestinal tone and motility produced by distension of some other part of the gut is called the intestino-intestinal inhibitory reflex. A similar response can be elicited by stimulation of the peritoneum, by distension of the ureters etc. Sympathetic intestino-inhibitory efferents mediate the reflex effects of these afferent systems (Johansson and Langston 1964) The physiological significance of the intestinal inhibition is not clear it is likely to be a nociceptive response in some situations but the profound effects of quite moderate stimuli (for instance the responses to low distension pressures in Fig 1 above) suggest that it may be in olved in the normal regulation of intestinal motility However an afferent nociceptive "back ground" activity is likely to prevail in the present experiments with abdominal surgery and this may have facilitated the responses to intestinal distension.

The main purpose of the present study was to elucidate the supraspinal control mechanisms influencing the spinal intestino-inhibitory reflexes. In a previous article (Johansson, Jonsson and Ljung 1965) it was shown that the intestino-intestinal inhibitory reflex could be blocked by topical stim lation in the lower med lla oblongata in agotomized cats. The present experiments strongly indicate that structures at this bulbar level exert a tonic restraint on the intestino-intestinal inhibitory reflex, whereas suprabulbar areas may ha th opposite influence.

Thus mesencephalic lesions resulted in a need for stronger stimuli to elicit intestinal nhibition compared to the control period. The increased basal tone and

motility of the gut seen in some experiments after decerebration can also be ascribed to elimination of a facilitating influence on reflex intestino-inhibitory "background" activity Spinalization following mesencephalic transections resulted in a conspicuous loss of intestinal tone and motility and in a decrease in the distension pressure required to elicit the intestino-intestinal inhibitory reflex. In the light of the previous study (Johansson, Jonsson and Ljung 1965) this can all be explained by a release of spinal intestino-inhibitory reflex activity from a tonic bulbar suppression probably originating in the area which gave strong reflex suppression when stimulated electrically The control mechanisms acting on the intestinal reflexes thus resemble or are identical with those described for the galvanic skin reflex by Wang and Brown (1956a, b) and by Wang, Stein and Brown (1956a, b)

After the intestines had been deprived of their extrinsic nerve supply by local anesthetic blockade of the periarterial nerves it was no longer possible to elicit an inhibitory response by distension. The marked increase in intestinal tone associated with the nerve blockade indicates that a considerable neurogenic inhibition was eliminated by the extrinsic denervation Evidently the intestino-inhibitory "background" reflexes exert a marked influence on intestinal tone and motility in the spinal animal.

This investigation was supported by grants from the Swedish Medical Research Council (B 67 14x 28-03) from the Medical Faculty University of Göteborg, From Air Force School of Aerospace Medicine under Contract AF 61(052) 732 through the European Office of Aerospace Research (OAR) United States Air Force, from U.S. Public Health Service (HE-05675-06) and from AB Hässle, Goteborg
Thanks are due to Mrs Gun Jidesten and Miss Brita Mellander for able technical assistance.

References

DOWNMAN C B B and B. A. McSWINEY Visceromotor reflexes in spinal animals *J Physiol.* (Lond) 1946. *105* 80—94

HOLMQVIST B and A. LUNDBERG, Differential supraspinal control of synaptic actions evoked by volleys in the flexion reflex afferents in alpha motoneurones. *Act physiol scand* 1961 *54* Suppl. 186 1—47

JOHANSSON B O JONSSON and B LJUNG Supraspinal control of the intestino-intestinal inhibitory reflex *Act physiol scand* 1965 *63* 442—449

JOHANSSON B. and J B LANGSTON Reflex influence of mesenteric afferents on renal, intestinal and muscle blood flow and on intestinal motility *Acta physiol. scand* 1964 *61* 400—412

WANG G H. and V W BROWN Changes in galvanic skin reflex after acute spinal transection in normal and decerebrate cats *J Neurophysiol.* 1956a. *19* 446—451

WANG, G. H and V W BROWN Suprasegmental inhibition of an autonomic reflex. *J Neurophysiol.* 1956 b *19* 564—572

WANG G H., P STEIN and V W BROWN Effect of transections of central neuraxis on galvanic skin reflex in anesthetized cats *J Neurophysiol* 1956 *19* 340—349

WANG, G. H., P STEIN and V W BROWN, Brainstem reticular system and galvanic skin reflex in acute decerebrate cats *J Neurophysiol* 1956 b. *19* 350—355

YAMAMOTO S. and K ARAKI Influence of brain stem on intra-abdominal pressure reflex of pelvic nerve origin. *Exp Neurol.* 1963 *7* 35—45

Acta physiol. scand. 1968 72. 205—219

From the Department of Pharmacology University of Lund, Sweden

The Mechanical Parameters of Myocardial Contraction Studied at a Constant Length of the Contractile Element

By

K. A. P Edman and E. Nilsson

Received 19 June 1967

Abstract

Edman, K. A. P and E. Nilsson. *The mechanical parameters of myocardial contraction studied at a constant length of the contractile element* Acta physiol. scand. 1968. 7 205—219

The time course of the active state, the force-velocity relation and the series compliance were investigated in isolated papillary muscles of the rabbit.—The force-velocity curve defined at precise length stage of the contractile element and at a given time after the stimulus could be fitted by Hill' equation. The shape of the curve remained constant during the activity period, indicating that the ability to produce motion and the ability to produce tension undergo parallel changes during a myocardial contraction cycle.—The active state, at a given length of the contractile unit, had a slow onset (1/3—1/2 of the entire activity period being needed for attainment of the maximum) and did not form a distinct plateau. The time course of the active state was critically dependent on the degree of extension of the contractile unit. Evidence is presented that the duration of the active state is not definitely programmed at the start of the contraction. Its final outcome is determined by the amount of shortening of the contractile element during the activity period.—The stiffness of the series elastic element, at a given tension, was independent of the intensity of the active state. This fact supports the view that the active force-producing structures in the cell do not contribute substantially to the series compliance of the cardiac muscle.

A useful model for analyzing the dynamics of muscular activity has been advanced by A. V. Hill (1938) in studies of the vertebrate skeletal muscle. According to this concept the muscle is assumed to function as an active contractile unit in series with an undamped elastic element. It is postulated that the contractile unit, when the cell is stimulated, is brought into an "active state" characterized by a capacity to produce motion or tension. Depending on whether the ends of the preparation are free or fixed the "active state" is manifested as an overall shortening of the muscle or as production of tension, in the latter case associated with stretch of the series elastic element produced by shortening of the contractile unit. According to the analytical approach devised by Hill the time course of the "active state" can be experimentally defined by recording the capacity of the muscle to shorten and to produce tension at selected times during the contraction. (For analysis of the

mechanics of the vertebrate skeletal muscle, see e.g. Katz 1939, Hill 1949, Wilkie 1950, Buchthal, Kaiser and Rosenfalck 1951, Abbott and Wilkie 1953, Ritchie 1954, Jewell and Wilkie 1958, 1960).

Attempts have been made during the last few years to explore the dynamics of the myocardial contraction using Hill's analytical model (Abbott and Mommaerts 1959, Mashima and Matsumura 1964, Brady 1965, 1966, Sonnenblick 1965, 1967 a). Certain features of the cardiac muscle function, however, makes an adequate study of the mechanical activity much more complicated in the myocardium than in the skeletal muscle. In the first place the cardiac muscle is unable to produce a fused tetanic contraction. This means that all measurements have to be performed at a defined time during the contraction in order to relate to a given state of activity of the muscle. Secondly, the mechanical output of the myocardium is critically dependent on the degree of extension of the contractile system. These two complicating factors have not been adequately controlled in previous studies of the heart muscle to enable a strict evaluation of the shape of the force-velocity curve relevant to a given instant after the stimulus and to a given length of the contractile unit. The problem is thus still unsettled as to how the capacity to shorten actively and the capacity to produce tension are changed relative to each other during activity of the cardiac muscle (Sonnenblick 1965, 1967 a).

The present investigation has been aimed at analyzing the dynamic parameters of the contraction of isolated rabbit papillary muscles by taking extreme care over factors in the recording technique which may affect the mechanical output. The experimental approach used has made it possible to define the time course of the myocardial active state at a precise length of the contractile element. The results have shown that the degree of extension of the cell is a determinant of both the maximal intensity of the active state and the duration of the mechanical activity. It will be demonstrated that the duration of the active state is not definitely scheduled when the contraction starts but may be affected by changes in length of the contractile unit during the contraction. A brief account of some of the results obtained in these studies have been given previously (Edman, Grieve and Nilsson 1966).

Methods

Dissection and mounting. Papillary muscles from rabbits were used. The rabbits (1.5–1.6 kg) were heparinized before being killed, and the heart was removed from the animal immediately after the slaughter and placed in well oxygenated Ringer solution. The papillary muscles were dissected from the right ventricle together with a piece of the ventricular wall. Great care was taken to reduce the elasticity in the connections between the muscle and the recording device. A small loop of platinum wire (0.15 mm thickness) was tied by means of silk thread to the tendon as closely as possible to the insertion of the muscle. Another platinum loop was tied to the piece of ventricular wall connected to the papillary muscle. For the experiment the muscle was mounted vertically in a jacketed thermostated Perspex bath. The lower end (the ventricular wall end) was attached to a tension transducer fitted in the bath; the upper end of the preparation (the tendon end) was fixed via a carefully straightened steel wire (0.25 mm thickness) to an isotonic lever.

Tension transducer. The tension was recorded by means of an RCA 5734 valve fitted into cylindrical block of Perspex which was plugged into the jacketed bath. The surface of the RCA-transducer exposed to the bathing solution was coated by grease (Joint grease K.W.S Martin Jaeger Geneva)

Isotonic lever. Length changes of the muscle were recorded by means of a duraluminium lever fitted on micro ball bearings. The equivalent mass of the lever at the point of attachment of the muscle was 115 mg and the static friction < 2 mg when the lever was freely suspended in air. In the presence of the damping fluids (see below) ordinarily used the natural frequency of the lever system (with the muscle suspended under a preload of 0.1 g) was 200 cycles per sec. The movements of the lever were recorded by a photo-electric device (Clairex Vacuum Photocell VS 42). Loading of the muscle was achieved by pplication of weights to the lever on the opposite side of the fulcrum. The hook to which the weights were applied was fitted on the lever by means of micro ball bearings. In order to minimize the inertia of the recording system caused by the load the ratio of the distances from the fulcrum to the points of attachment of the load and the muscle was 1 10. As further means of reducing the moment of inertia the *load was attached to the lever via suitable elasticity a coil spring or a rubber band, depending* on the load used. By these arrangements the load could be considered as pure force acting on the muscle. A dash-pot containing silicone oil and placed on the same side of the fulcrum as the muscle was used to damp oscillations of the lever during quick-release-recordings. A 1 mm wide duraluminium peg provided with thin metal disk at its distal end extended from the lever into the damping fluid. The degree of damping was varied by using silicone oils of different viscosity (Silicone fluid MS, viscosity 200—1250 Cs)

An upper stop screw controlled the resting length of the muscle. In order to eliminate the cohesive force between the contact surfaces of the lever and the stop screw stylus of diamond was mounted on the lever opposite the stop. The lever could be locked against the upper stop screw by means of releasable catch fitted on telephone relay. This stop was synchronized with the stimulating pulse and could be withdrawn at any pre-set moment during the contraction to enable isotonic shortening of the muscle after an initial isometric phase. The amount of shortening of the preparation could be controlled by third stop.

Stimulation. The muscle was stimulated by an assembly of platinum wire (0.3 mm diam.) electrodes passing transversely across the longitudinal axis of the preparation. The individual electrodes of the assembly were placed 0.5 mm apart from each other and arranged in pairs of anodes and cathodes. In this way the muscle could be stimulated two sites simultaneously. The distance of propagation of the action potential within the preparation ould thus be limited to 1.0 mm. This implies, assuming a propagation velocity of approximately 1 m/sec (Hoffman and Cranefield 1960) that all parts of the preparation were excited about 1 msec after the stimulus was applied. Supramaximal pulses of 2 msec duration (rise time < 0.1 msec) were used for stimulation.

Recording. The signals from the tension transducer and from the photo-cell of the isotonic lever were displayed on Tektronix 502 A oscilloscope. The first derivat of the output from the two transducers was obtained by feeding the signals into an RC-circuit time constant 0.3 msec). One of the beams of the oscilloscope was switched by means of an lectronic oscillator (frequency 1250 cps) to enable simultaneous recordings of the signals of the isotonic lever the RCA 5734 alv and also the differentiated form of either of the signals. The oscilloscope traces were recorded photographically using Consor oscilloscope camera.

Determination of the stray compliance. For determination of the stray compliance of the recording device the same connections between the tension transducer and the isotonic lever were used as during an ordinary experiment. The papillary muscle was replaced by an 8 mm long metal wire (0.3 mm thickness) which was tied with silk threads to the platinum loops used for mounting of the muscle during the experiments. The degree of extension of the connections between the two transducers (includin the bending of the isotonic lever and the peg of the RCA 5734 alv) was recorded by the photo-cell of the isotonic lever he load wa recorded by the tension transducer. The magnitude of the stray compliance t different loads is illustrated in Fig. 8

Resting length. A given overall resting length, corresponding to preload of 100 mg was used throughout an experiment. The resting length was determined as the distance between the insertions of the muscle to the upper tendon and the entricle wall. The distance was read against millimeter scale situated closely to the muscle in the bath. The insertion of the papillary muscle to the ventricular wall is not distinct and the true functional length of the muscle proper probably deviated from the measured lengths by ± 5 per ent

Solution. A Ringer's solution of the following composition was used (mM): NaCl 120, KCl 5, $NaHCO_3$ 25, NaH_2PO_4 1.5, $CaCl_2$ 2.0, Glucose 10.

Glass distilled water was used for washing of the glassware and for the preparation of the solutions. All the chemicals used were of analytical grade.

Results

There is reason to believe (Abbott and Mommaerts 1959) that the contractile system of the myocardium, similar to the situation in the skeletal muscle (Hill 1938) may be considered equivalent to an active contractile unit in series with an elastic component. The present analysis of the myocardial contraction is based on this conception. Interference from parallel elastic elements are considered negligible in this investigation, since the experiments have been performed at a low resting tension.

A. The time course of the myocardial active state

The intensity of the active state at different times after the stimulation was determined by using a similar approach to that devised by Jewell and Wilkie (1960) in studies of skeletal muscle. The papillary muscle, suspended between a tension transducer and an isotonic lever (see Methods) was paced to contract isometrically at a constant frequency, the lever being fixed by means of a relay-operated catch. During selected contractions, about 30 sec apart, the catch was quickly withdrawn at a pre-set time after the stimulus, and the muscle was allowed to shorten isotonically against a small load, 100 mg. Oscilloscope records from a typical release contraction are presented in Fig. 1 showing simultaneous changes in length (A), shortening velocity (B) and tension (C) following upon the quick release. The shortening took part in two steps. The initial rapid length change involved movements of both the series elastic element and the contractile unit, the angle of the

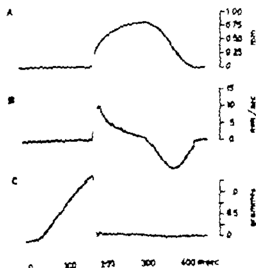


Fig. 1. Oscilloscope records of release experiment. A. Shortening. B. Velocity of shortening (electrical differentiation) and C. Tension. Stimulation signal at zero time. Temperature 29.5° C. Contraction frequency: 30 per min.

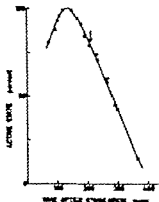


Fig. 2. Active state curve of rabbit papillary muscle at 72 contractions per min and 29 C. Velocity data used for construction of the curv refer to the length of the contractile unit existing t the maximum of the isometric twitch. Arrow indicates time for the isometric peak twitch tension. Ordinate I tensity of the active state, percent of maximum. Abscissa Time after stimulus.

shortening curve denoting the time at which the series elastic component had equilibrated with the load. The subsequent, less rapid phase thus represented the shortening of the contractile unit alone. The velocity of shortening during the second phase at a gi en decrease in length of the preparation, was used as an index of the active state at that instant. By releasing the muscle at different moments after the stimulus the time course of the active state, at a given length of the contractile unit, could be defined.

The fact that constant load was used in the release contractions implies that the degree of extension of the series elastic component was the same during the second phase of the shortening in all cases. A given overall length of the preparation during the second phase thus orresponded to given length state of the active unit.

Fig 2 illustrates the shape of the active state curv of the rabbit papillary muscle at 29° C and a contraction frequency of 72 per minute. The velocity data on which the curve is based, refer to the length of the contractile unit existing at the peak of the isometric twitch. The arrow in the diagram indicates the time for the isometric peak twitch tension. The active state, as can be seen did not form a distinct plateau the activity increased gradually to a maximum and passed smoothly over to the decay phase The rise of the activity was slow 1/3—1/2 of the entire activ state time being used for attainment of the maximum.

The intensity of the active state at various times after the stimulation w critically dependent on the length of the contractile unit, as is shown in Fig 3 Illustrated are three active state curves derived from elocity measurements at three different degrees of shortening of the contractile unit using the same oscilloscope records. Shortening of the contractile unit caused 1 a reduction of the amplitude of the active state curv and 2 a decrease of the duration of the acti state No distinct change of the time for attainment of maximal ctivity was observed Table I summarizing the results of 7 expts. present quantitativ data f the changes of the peak activity and the time for decay of the activ stat to 50 per cent of its peak value in consequence of shortening of the muscle The length changes are expressed

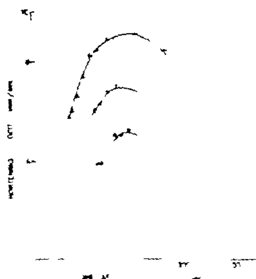


Fig. 3. Active state curves of rabbit papillary muscle defined at three different lengths of the contractile unit. Measurements from the same oscilloscope records in all curves. Lengths expressed in percent of the resting muscle length at 100 mg preload. Δ 96 percent, ○ 92 percent, ● 88 percent. Contraction frequency 45 per min. Temperature 29 C.

Table 1

Date of experiment	Temp. C.	Contraction frequency min	Length of the contractile system (per cent of resting muscle length)	Maximal intensity of the active state units	Time from stimulus to half decay of the active state msec
12.10.—6	2	45	9	1.00	480
			88	0.	425
4.11.—4	2	45	9	1.00	373
			88	0.57	323
9.11	2	4	9	1.00	3 4
			88	0.57	292
2. —4.5	30	3	94	1.00	266
			89	0. 6	24
2. 5 —6	3	3	91	1.00	254
			83	0.52	240
21.5 —4	30	3	92	1.00	314
			87	0.51	294
21. —6	31	3	1	1.00	336
			83	0.40	308

as percent of the resting length of the preparation and the maximal intensity of the active state recorded at the greater length is taken as unity. The results show that by 5—8 per cent shortening of the muscle (at approximately 90 per cent of its resting length) the maximal intensity of the active state was reduced almost to one half. The time from the stimulus to 50 per cent decay of the active state was reduced on the average by 38 msec. The conclusion of these findings is that the time course

of the myocardial active state is not solely determined by the conditions of the muscle at the moment of stimulation, both the maximal intensity of the active state and the duration of the mechanical activity may be affected by changes in length of the muscle during the contraction.

The active state curve, as determined in the present study refers to ideal isometry of the contractile unit, i.e. all measurements have been carried out at a precise length state of the muscle. It is clear that the active state curve so derived is not a quantitative index of the activity during the conventional isometric twitch response, which involves a substantial amount of intrinsic shortening of the muscle (Section C). In order to define the active state course during an ordinary (pseudo-)isometric contraction, or during an isotonic twitch, due allowance would thus have to be made for the actual length of the contractile element at each instant during the contraction.

In previous analyses of the myocardial active state (Abbott and Mommaerts 1959 Mashima and Matsumura 1964 Brady 1965 1966, Sonnenblick 1965 1967 a) there was no control for the intrinsic" length changes of the muscle during the isometric contraction. This factor of uncertainty in the earlier studies, as in fact was pointed out by Brady (1966) and Sonnenblick (1967 a) greatly limited the possibility to evaluate, quantitatively the kinetics of the active state in the cardiac muscle. It is evident from the present results, however that the general characteristics of the active state curve as observed in the previous investigations, i.e. the slow onset and the lack of a distinct plateau, do hold true also at a constant contractile element length.

B. *The force-velocity relation*

For a complete analyses of the active state it was essential to find out whether or not the force-velocity relation undergoes any fundamental change during the activity period. The following experiments were performed to investigate this point by determining the force-velocity curve at different times during the contraction cycle. Before presenting these experiments the general shape of the force velocity curve of the cardiac muscle will be described.

The speed of shortening at various loads was determined by releasing the muscle at a selected time during an isometric contraction. The velocity during the second shortening phase after the release (*cf.* Fig. 1) at a given time after the stimulus, was used in each case. In this way the speed of shortening of the contractile unit could be defined over a wide range of loads at a fixed time in the activity period and at a nearly constant length of the contractile unit. The difference in length of the active component between the velocity measurements performed at the smallest and the greatest loads in a given series was less than 1 per cent of the resting muscle length. Fig. 4 illustrates the force-velocity curve of a rabbit papillary muscle at 27.5 C and a contraction frequency of 45 per min. The experimental data refer to measurements carried out at the time for the isometric peak twitch tension. Under these conditions the intersection of the curve with the abscissa, i.e. the mechanical

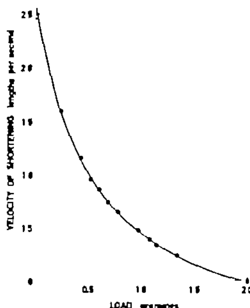


Fig. 4 Force-velocity curve of rabbit papillary muscle at 45 contractions per min and 27.5 C. All velocity data refer to the length of the contractile unit existing at the peak of the isometric twitch. The tension at zero velocity is the isometric twitch tension. Curve fitted for (P + 0.50) (V + 0.66) = 1.65

output at zero velocity is defined by the isometric peak twitch tension. The experimental data can be fitted by Hill s equation

$$(P + a)\ V = (P - P)\ b$$

in which P is the load used, P_0 the maximal capacity to develop tension at the state of activity considered, V the speed of shortening and a and b constants with dimensions of tension and velocity respectively. The curve fitted to the experimental data in Fig. 4 gives a value for $V_{max} = 2.6$ lengths/sec and $a/P_0 = 0.25$. It should be pointed out that the hyperbolic shape of the force-velocity curve as described here does only apply if the measurements are made at a given functional state of the muscle, i.e. at a constant length of the active component and a constant time during the activity period. Inconsistency of the length of the contractile unit between the individual measurements exceeding 2 per cent of the resting muscle length (cf. Table I, section A) would cause a substantial distortion of the curve.

Seven experiments were carried out in which the shape of the force-velocity curve was defined at two or more different instants during the activity period. The results of three typical experiments are illustrated in Fig. 5. The curves have been drawn according to Hill's equation using the same constants for *a* and *b* in the respective experiments. As can be seen the experimental data fit the curve reasonably well at the various instances investigated. The force-velocity curve undergoes a continuous shift during the activity period. The *shape* of the curve, however, and hence the ratio between V_{ax} (zero load) and P_0, remains practically constant over the dif-

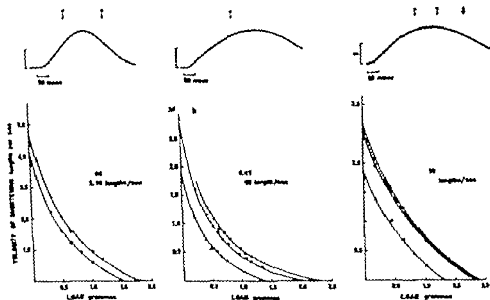


Fig 5 Force-velocity curves (lower diagrams) defined at different times during the activity period as indicated by arrows in the isometric myograms (upper diagrams) Curves drawn according to Hill's equation for values of nd *b* given in the respective diagrams. The velocity values t given load refer to the same length of the active unit in all curves of the same experiment. The variation in length of the activ unit between the measurements at the smallest and the greatest loads was < 0.73 percent of the resting muscle length in a, < 0.5 percent in b nd < 1 0 percent in c.
Contraction frequency and temperature a. 45/min. 31 C b 45/min 27.5 C c. 45/min 26 3 C

ferent time intervals studied. The results thus support the view that the ability of the contractile element to produce motion and the ability to develop tension run parallel during a contraction cycle The active state curve defined in terms of shortening velocity according to the approach used in the previous section, may therefore also be considered representative for the capacity to produce tension under the same experimental conditions.

C. *Th serie mphase*

The stiffness of the series elastic element was determined as the drop in tension (ΔP) that occurred in response to a quick controlled release (ΔL) at gi en tension levels (P) during isometric contraction of the muscle The release was produced by withdrawal of the catch, by which the isotonic lever was clamped during isometric recording, and the lever was allowed to move to another pre-set stop In this way t was possible to produce a precise length change of the muscle at any moment dur ing the contraction. The shortening amounted to approximately one percent of the resting muscle length in the different experiments and was attained within 5 msec after removal of the catch. Fig 6 is a reproduction of typical oscilloscope records illustrating the simultaneous changes in length and tension that follow upon the

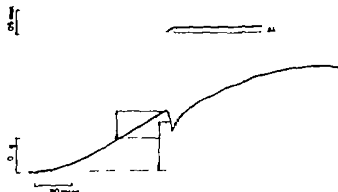


Fig. 6. Oscilloscope records of quick release for determination of the stiffness ($\Delta P/\Delta L$) of the series elastic element of rabbit papillary muscle. The amount of release (upper record) and the resulting tension drop (lower record) recorded on the same time base. The way of measuring ΔL, ΔP and the mean tension (P) during the length change is illustrated by dotted lines.

release. The tension response (ΔP) of the undamped series elastic element was derived, as is demonstrated in Fig. 6, by extrapolation from the isometric myogram back to the instant of release of the muscle. The value for P was taken as the mean tension of the series elastic component during the length change. Fig. 7 illustrates the results of two different experiments, in which $\Delta P/\Delta L$ was determined at various tension levels during the rising and falling phases of the contraction. It can be seen that the stiffness of the series elastic component increased in direct proportion to

Fig. 7. The stiffness of the series elastic element (ordinate) of two rabbit papillary muscles at different tensions (abscissa). Measurements carried out both during the rising phase (open symbols) and during the decay phase (filled symbols) of the isometric twitch. Contraction frequency 36/min in both experiments.
Temperature: a. 31 °C; b. 23 °C.
The root mean square difference between observed data and regression is: a. 0.48 g/mm; b. 0.29 g/mm.

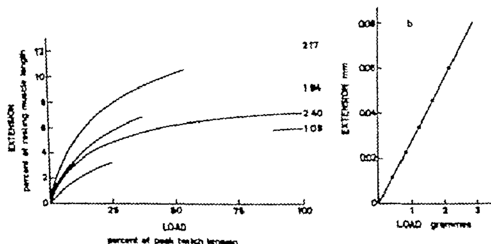


Fig. 8. a. Load-extension curves of the series elastic component in four papillary muscles. Abscissa: tension beyond preload in percent of peak twitch tension. Ordinate: extension in percent of total resting muscle length. Isometric peak twitch tension (grammes) indicated at respect curve. Curves corrected for the stray compliance of the recording device. Temperature 30° C.
Contraction frequency: 30—36/min.
b. Stray compliance of the recording device. For details of the measurements, see Method.

the tension. No difference existed between measurements performed during different phases of the mechanical activity of the muscle. The stiffness ΔP/ΔL, at a given P was thus the same irrespective of whether the measurement was made during the rising phase of the contraction or during the relaxation. This means, considering the great difference in the intensity of the active state that exists at equivalent tension levels during rise and decay of the isometric contraction (*cf.* Fig. 2) that the mechanical properties of the series elastic element were virtually unaffected by changes in the functional state of the contractile unit during the activity. This conclusion is in full accord with the fact, to be described in a following paper, that the series compliance was uninfluenced by an inotropic intervention of the myocardium.

Knowing the relationship between ΔP/ΔL and P, the length-tension curve for the series elastic element can be derived. According to Fig. 7 ΔP/ΔL = k · P + C, in which k is the slope of the line and C the intercept with the ordinate. After integration the following equation relating length and tensions is obtained: P = a (e^{kL} — 1) in which a is the pre-load of the muscle and L the elongation of the series elastic component beyond the length attained at rest of the muscle. The constant C has been neglected.

Fig. 8 a demonstrates the length-tension curves of the series elastic component determined in 4 different papillary muscles. The curves have been corrected for the stray compliance that existed in the recording device and the connections with the muscle. The magnitude of the stray compliance is illustrated separately (Fig. 8 b). The results show that even at strict isometry for the muscle as a whole there were

considerable "intrinsic" length changes during contraction. Under the conditions used the series elastic element was stretched by approximately 6—12 per cent of the resting muscle length, with a corresponding shortening of the contractile unit, at the maximum of the isometric contraction.

The series elastic element of the isolated cat papillary muscle has been investigated by Sonnenblick (1964) using an indirect experimental approach and, in a more recent study by the same author (Sonnenblick 1967 b) by means of a modified version of the quick-release technique devised by Wilkie (1956) The series compliance of the cat papillary muscle preparation could be reduced to 4—5 per cent of the resting muscle length at the peak of the isometric twitch. In accordance with the results obtained in the present investigation the stiffness of the series elastic element was only dependent on the actual tension it was not markedly affected by changes in the intensity of the active state.

Discussion

The time course of the active state of the myocardium. The degree of activity of the contractile system of the rabbit papillary muscle was analyzed by determining the capacity of the muscle to produce mechanical output at different times after the stimulation. The approach used enabled a detailed study of the time course of the activity at a given length state of the contractile unit. It has been demonstrated that the ability of the contractile element to shorten actively and the ability to develop tension undergo virtually parallel changes during the contraction cycle. The original concept of the "active state" (Hill 1938) providing a common parameter for tension and shortening velocity is therefore applicable in the analysis of the myocardial function.

The time course of the active state of the myocardium (also see Brady 1965, 1966 Sonnenblick 1965 1967 a) differs in several important respects from that of the skeletal muscle (Edman and Grieve 1966 Edman, Grieve and Nilsson 1966) 1 Whilst the rising phase of the active state in the skeletal muscle cell is very rapid, there is a slow rise of the activity in the cardiac muscle, 1/3—1/2 of the total active state time being used for attainment of the maximum 2 The maximal intensity of the active state of the myocardium, defined at a given length of the contractile unit, is a variable It may be changed over a wide range by for instance, an alteration of the contraction frequency (Edman, Grieve and Nilsson 1966) 3 The active state curve of the myocardium does not have a distinct plateau, the activity passes from rise to decay forming a smooth maximum. Taken together these characteristics of the myocardial active state provide clear evidence that the contractile system in the heart muscle as studied in the papillary muscle preparation, does not attain mechanical saturation during a period of activity This is in contrast to the situation in the skeletal muscle cell in which the active state during a single cycle attains the very maximum of which the contractile system is capable at the sarcomere length considered.

The length dependence of the active stat The analysis has shown that the active state course of the myocardium is critically dependent on the degree of extension of the contractile system. Both the *maximal intensity* of the active state and the *time relation* of the rise and decay of the activity are affected by relatively small changes in length of the muscle The results have indicated that the time course of the active state does not depend solely on the degree of extension of the muscle at the onset of contraction it may be affected continuously during the contraction by an alteration in the length of the contractile unit. This is an interesting finding because it shows that the time course of the active state is not definitely programmed when the muscle starts to contract. The eventual outcome of the active state course — the peak intensity as also the *du ation* of the activity — is dependent on the amount of shortening of the contractile unit during the contraction.

The length dependence of the active state curve is not unique to the cardiac muscle As was recently demonstrated in isolated skeletal muscle fibres of the frog (Edman and Kiessling 1966 Edman 1966) the duration of the active state is progressively reduced also in this preparation by shortening of the cell.

Th activator m hanism of the citation-contraction p oc Abundant data support the view that the activity of the contractile element is controlled by means of an activator agent, probably calcium, released or transported into the myofibrillar space in consequence of the excitation of the cell membrane. (For references see recent review articles by Hasselbach 1962, Brady 1964 Huxley 1964 Klaus and Lüllman 1964 Edman 1965 Sandow 1965 Nayler 1966) The details of the mechanisms governing the release and the removal of the activator are still incompletely known. The active state curve defined at a precise length of the contractile system, provides a relevant guide in the analysis of the kinetics of the activator in the lectromechanical coupling The differences in the shape of the active state curve in the skeletal muscle and the myocardium (see previously) would seem to imply that the rate of release (or inflow) and the rate of elimination of the act vator agent are different n the two types of muscle The activator concentration in the nterior of the myocardial cell may thus be assumed to be built up slowly w thout reaching the level for mechanical saturation of the contractile element The data from the skeletal muscle fibre on the other hand, suggest that there is a ery rapid increase of the activator concentration, and that, furthermore the concentration exceeds the level for maximal activation of the contractile system. The length dependence of the duration of the active state brings a new interesting aspect upon the act ator mechanism in the lectromechanical coupling It suggests that the metabolism of the activator i.e the rate of release and/or the rate of limination of the act ator is dependent on the length state of the cell.

The serie elastic lement The series elastic lement of the papillary muscle beha es a a passi entity its mechanical properties being uninfluenced by the functional state of the contractile unit. This conclusion is based on the following facts

1 The rigidity of the series elastic component, at a gi en tension, the same during

the rising phase of the twitch and during relaxation, i.e. at times when the intensity *of the active state is greatly different.*

2. The load-extension curve of the series elastic element is unaffected during an inotropic intervention of the myocardium as produced by altered pacing of the muscle (Edman and Nilsson to be published)

The extension of the series elastic component of the rabbit papillary muscle on the height of the isometric contraction amounts to 6—12 per cent of the total resting length of the muscle. Only 1/10 of this value can be accounted for by stray compliance in the mechanical system. The anatomical distribution of the series elasticity in the papillary muscle preparation has not been studied quantitatively. A great portion of the elastic compliance is no doubt localized in the tendon and the piece of the ventricle wall used for attachment of the preparation in the recording device. Another portion probably resides in the papillary muscle itself due to the particular architecture of the cardiac tissue. The non-parallel arrangement of the fibres in the myocardium would allow a certain degree of shortening of the individual elements, even when the ends of the preparation are carefully fixed, and would thus introduce an extra series compliance into the system. Part of the series elastic element might have its morphological counterpart inside the cell, as pointed out previously (Hill 1950, Jewell and Wilkie 1958, Sonnenblick 1964, 1967 b). It would seem clear from the present results, however, that the active, force-producing structures in the cell do not contribute to any substantial degree to the series compliance of the cardiac muscle.

The authors wish to express their gratitude to Miss L. Flork, Miss K. Koch and Miss M. Olsson for their technical assistance during this investigation. This work was supported by research grants from the Swedish Medical Research Council (Project No. 14x-184), the Swedish National Association against Heart and Chest Diseases and the Medical Faculty of the University of Lund.

References

ABBOTT, B. C. and W. F. H. M. MOMMAERTS, A study of inotropic mechanisms in the papillary muscle preparation. *J. gen. Physiol.* 1959. *42.* 533—551.

ABBOTT, B. C. and D. R. WILKIE, The relation between velocity of shortening and the tension-length curve of skeletal muscle. *J. Physiol.* (Lond.) 1953. *120.* 214—223.

BRADY, A. J., Excitation and excitation-contraction coupling in cardiac muscle. *Ann. Rev. Physiol.* 1964. *26.* 341—356.

BRADY, A. J., Time and displacement dependence of cardiac contractility: problems in defining the active state and the force-velocity relation. Fed. Proc. 1965. *24.* 1410—1420.

BRADY, A. J., Onset of contractility in cardiac muscle. *J. Physiol.* (Lond.) 1966. *184.* 560—580.

BUCHTHAL, F., E. KAISER and P. ROSENFALCK, The rheology of the cross striated muscle fibre. *Dan. Biol. Medd.* 1951. *21.* No. 7. 1—318.

EDMAN, K. A. P., *Drugs and properties of heart muscle. Ann. Rev. Pharmacol.* 1965. 5. 99—118.

EDMAN, K. A. P., The time course of the active state in the vertebrate skeletal muscle in relation to sarcomere length. Symposium on muscle. Budapest 1966. *Hung. Acad. Sci.* In press.

EDMAN, K. A. P. and D. W. GRIEVE, The mechanical parameters of the contraction of single muscle fibres of the frog. *J. Physiol.* (Lond.) 1966. *184.* 21—22 P.

EDMAN, K. A. P., D. W. GRIEVE and E. NILSSON, Studies of the excitation-contraction mechanism in the skeletal muscle and the myocardium. *Pflügers Arch. ges. Physiol.* 1966. *290.* 320—334.

EDMAN, K. A. P. and A. KIESSLING, The time course of the active state of the frog skeletal muscle in relation to the sarcomere length. *Acta physiol. scand.* 1966. 68. Suppl. 277. 41.

HASSELBACH W. Muskelphysiologie. Die Kopplung chemischer und mechanischer Reaktionen während Kontraktion und Erschlaffung des Muskels. *Fortsch. Zool.* 1962, *15* 1—91
HILL, A. V. The heat of shortening and the dynamic constants of muscle *Proc. Roy. Soc. B* 1938 *126* 136—195
HILL, A. V. The abrupt transition from rest to activity in muscle. *Proc. Roy. Soc. B* 1949 *136* 399—420
HILL, A. V. The series elastic component of muscle. *Proc. Roy. Soc. B* 1950 *137* 273—280
HOFFMAN, B. F. and P. F. CRANEFIELD *Electrophysiology of the heart*. New York McGraw Hill, 1960
HUXLEY A. F. Muscle. *A. Rev. Physiol.* 1964 *26* 131—152.
JEWELL, B. R. and D. R. WILKIE, An analysis of the mechanical components in frog striated muscle. *J. Physiol.* (Lond.) 1958, *143* 515—540
JEWELL, B. R. and D. R. WILKIE, The mechanical properties of relaxing muscle *J. Physiol.* (Lond.) 1960 *152* 30—47
KATZ, B., The relation between force and speed in muscular contraction. *J. Physiol.* (Lond.) 1939 *96*. 45—64
KAUL, W. and H. LÜLLMANN, Calcium als intracellulärer Überträgersubstanz und die mögliche Bedeutung dieser Mechanismus für die pharmakologische Wirkungen *Klin. Wschr.* 1964 *42* 253—59
MASHIMA, H. and M. MATSUMURA, The effect of temperature on the mechanical properties and action potential of isolated frog ventricle *Jap. J. Physiol.* 1964 *14* 422—438
NAYLER, W. G. Influx and efflux of calcium in the physiology of muscle contraction. *Clin. Orthop.* 1966 *46* 157—182
RITCHIE, J. M., The duration of the plateau of full activity in frog muscle. *J. Physiol.* (Lond.) 1954 *124* 605—61
SANDOW A. Excitation-contraction coupling in skeletal muscle *Pharmacol. Rev.* 1965 *17* 265—320
SONNENBLICK, E. H., Series elastic and contractile elements in heart muscle changes in muscle length *Amer. J. Physiol.* 1964 *207* 1330—1338
SONNENBLICK, E. H. Determinants of active state in heart muscle Force, velocity instantaneous muscle length, time *Fed. Proc.* 1965 *24* 1396—1409
SONNENBLICK, E. H., Active state in heart muscle. Its delayed onset and modification by inotropic agents *J. gen. Physiol.* 1967a. *50* 661—676
SONNENBLICK, E. H., Series Elasticity in heart muscle its relation to contractile element velocity and proposed muscle models. *Circulat. Res.* 1967b *20* 112—123
WILKIE, D. R., The relation between force and velocity in human muscle. *J. Physiol.* (Lond.) 1950 *110* 249—280
WILKIE D. R., Measurement of the series elastic component at various times during a single muscle twitch. *J. Physiol.* (Lond.) 1956 *134* 527—530
WILKIE, D. R. Muscle *A. Rev. Physiol.* 1966 *28* 17—38

Acta physiol. scand. 1968. 72. 220—233

From the Department of Physiology University of Göteborg, Sweden

Changes in Cardiac Output upon Stimulation of the Hypothalamic Defence Area and the Medullary Depressor Area in the Cat

By

B. Folkow B. Lisander, R. S Tuttle[1] and S. C. Wang[2]

Received 22 June 1967

Abstract

Folkow B., B. Lisander R. S Tuttle and S C. Wang. *Changes in cardiac output upon stimulation of the hypothalamic defence area and the medullary depressor area in the cat* Acta physiol. scand. 1968. 72 220—233

The hypothalamic defence area and the bulbar depressor area, with related hypothalamic sympatho-inhibitory structures, were topically stimulated while changes in cardiac output (C. O) heart rate stroke volume, central venous pressure and peripheral blood flow were studied in anaesthetized-curarized cats and dogs. Defence area stimulation induced up to [illegible]-fold increases in C. O and [illegible]p to threefold increases [illegible] calculated work load for the left [illegible]le. The major part of the C. O increase was directed to the skeletal muscles, [illegible] [illegible] [illegible]holinergic [illegible]asodilatation increased flow [illegible]p to fivefold. Gastrointestinal and renal blood [illegible] were promptly reduced and since this neurogenic flow reduction slightly preceded muscle [illegible]asodilatation, the C. O increase could be preceded by a very brief phase of C. O decrease. [illegible] adrenergic blockade could reduce the threefold increase in left ventricular work load by 50—60 per cent by eliminating the sympathetic dri[illegible] on the heart. Depressor area stimulation produced an often profound reduction in C. O sometimes preceded by [illegible] brief C. O increase. Flow resistance fell, especially so in sk[illegible]letal muscles, where an initial flow increase coincided with the transient C O increase further blood was pooled within the capacitance sections. Blood loss or raised [illegible]enous transmural pressure greatly potentiated these [illegible]hanges so that imminent cardiovascular collapse could be induced by this central depressor stimulation. The mechanisms involved in the mentioned, diametrically opposite cardiovascular patterns are discussed.

It is well known that in cats and dogs, stimulation of a restricted hypothalamic region the defence area can induce a drastic autonomic response pattern involving a cholinergic [illegible]asodilation in skeletal muscles with excitation of the adrenergic fibres to most other sections of the cardiovascular system (*cf* Uvnäs 1960 *Folkow et al.* 1963) Experiments in various species including man indicate that virtually identical vasomotor changes take place during emotional "stress" thus this hypothalamic area is generally assumed to be involved in emergency reactions. Studies

Present addresses.

[1] Masonic Medical Research Laboratory Utica, N.Y. 13501 USA. PHS NB 03501—02

[2] Department of Pharmacology College of Physicians & Surgeons of Columbia University 630 West 168th Street, New York N.Y. 10032, USA.

on unanesthetized cats (Abrahams *et al.* 1961) further make it clear that these topically evoked autonomic changes are normally paralleled by an alerting response that can escalate into attack or escape behaviour and that hormonal activation also takes place (*cf.* Folkow *et al.* 1967). There is thus formed a well integrated response pattern involving all three efferent systems.

Stimulation of the medullary depressor area, on the other hand, leads to profound inhibition of tonic sympathetic activity of the entire cardiovascular system, and in addition, to an increased vagal discharge to the heart. This bulbar area normally appears to relay both the depressor influences emanating from cortico-hypothalamic structures, as well as those depressor influences arising peripherally from the cardiovascular baroreceptors and the so-called "somatic depressor fibres" (*cf.* Johansson 1962). It has been postulated (Löfving 1961) that this depressor response when induced from cortico-hypothalamic structures via the medullary depressor area, might be involved in emotional fainting in man and in "playing dead" reactions in some species, especially since it appears to be associated with suppression of somatomotor activity as well. Thus this depressor reaction would represent another type of apparently protective response which, however, in most of its expressions is diametrically opposite to the defence reaction.

The great majority of studies regarding these autonomic responses to central nervous stimulation has been directed towards the adjustments of the different vascular beds. Despite the availability of some very interesting data concerning the direct nervous action on the heart during stimulation of the defence area of the cat (Rosén 1961) and of corresponding hypothalamic structures in the dog (Rushmer 1962), there is very little information concerning the influence of the defence reaction and of the depressor response on cardiac output and central hemodynamics. It was therefore considered of interest to compare the impact of the two response patterns mentioned above on cardiac output and on central hemodynamics. Some of the present findings have earlier been presented in a brief preliminary report (Folkow *et al.* 1966).

Methods

Experiments were performed on 34 cats of both sexes, weighing 2.0—6.0 kg and on 3 dogs weighing 5—10 kg. After induction with ether light anesthesia was maintained by administration of chloralose 50—90 mg/kg, pentobarbital sodium 30—40 mg/kg or chloralose-urethane mixture 25 and 200 mg/kg, respectively. Free airway passage was secured by inserting a tracheal cannula.

For topical stimulation in the brain the animal's head was fixed in a Horsley-Clarke apparatus and a sharp monopolar electrode, insulated except at the very tip, was used. A Grass S5 stimulator delivered square pulses with an intensity of 1—6 V and a duration of 1—3 msec. The frequency was varied within wide limits (20—100/sec).

The defence area was reached stereotaxically through a small burr hole in the skull. When the medullary depressor area was to be stimulated, the muscles of the posterior part of the neck were divided in the midline, parts of the occipital bone were removed and the dura opened, and the cerebellum was gently lifted or partly removed by suction to provide free access to the floor of the fourth ventricle.

At the conclusion of each experiment, the position of the electrode was checked by staining of the electrode track, followed by gentle dissection and inspection of the area, if necessary under magnification.

Heparin was given as an anticoagulant. Blood pressure was measured through a catheter

Acta physiol. scand. 1968. 72. 220—233

From the Department of Physiology, University of Göteborg, Sweden

Changes in Cardiac Output upon Stimulation of the Hypothalamic Defence Area and the Medullary Depressor Area in the Cat

By

B. Folkow, B. Lisander, R. S. Tuttle[1] and S. C. Wang[2]

Received 22 June 1967

Abstract

Folkow, B., B. Lisander, R. S. Tuttle and S. C. Wang. *Changes in cardiac output upon stimulation of the hypothalamic defence area and the medullary depressor area in the cat.* Acta physiol. scand. 1968. 72. 220—233.

The hypothalamic defence area and the bulbar depressor area, with related hypothalamic sympatho-inhibitory structures, were topically stimulated while changes in cardiac output (C. O.), heart rate, stroke volume, central venous pressure and peripheral blood flow were studied in anaesthetized-curarized cats and dogs. Defence area stimulation induced up to twofold increases in C. O. and up to threefold increases in calculated work load for the left ventricle. The major part of the C. O. increase was directed to the skeletal muscles, here the cholinergic vasodilatation increased flow up to fivefold. Gastrointestinal and renal blood were promptly reduced and since this neurogenic flow reduction slightly preceded muscle vasodilatation, the C. O. increase could be preceded by a very brief phase of C. O. decrease. β-adrenergic blockade could reduce the threefold increase in left ventricular work load by 50—60 per cent by eliminating the sympathetic drive on the heart. Depressor area stimulation produced an often profound reduction in C. O., sometimes preceded by a brief C. O. increase. Flow resistance fell, especially so in skeletal muscles, where an initial flow increase coincided with the transient C. O. increase; further, blood was pooled within the capacitance section. Blood loss or raised venous transmural pressure greatly potentiated these changes so that imminent cardiovascular collapse could be induced by this central depressor stimulation. The mechanisms involved in the mentioned, diametrically opposite cardiovascular patterns are discussed.

It is well known that in cats and dogs, stimulation of a restricted hypothalamic region, the defence area, can induce a drastic autonomic response pattern involving a cholinergic vasodilation in skeletal muscles with excitation of the adrenergic fibres to most other sections of the cardiovascular system (*cf.* Uvnäs 1960, Folkow *et al.* 1963). Experiments in various species including man indicate that virtually identical visceromotor changes take place during emotional stress; thus this hypothalamic area is generally assumed to be involved in emergency reactions. Studies

Present addresses:

1 Masonic Medical Research Laboratory, Utica, N.Y. 13501, USA. PHS NB 03501—0.

2 Department of Pharmacology, College of Physicians & Surgeons of Columbia University, 630 West 168th Street, New York, N.Y. 10032, USA.

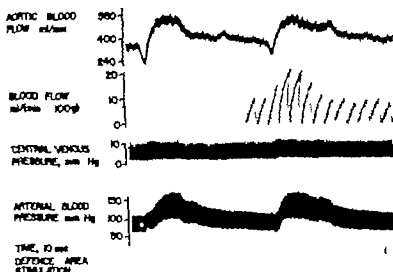


Fig. 1 Dog 4 75 kg Effects of two periods of stimulation in the hypothalamic defence area. A result of the first stimulation period (2 msec duration, 5 V 80/sec) the muscle blood flow instead of falling in separate drops through the drop chamber passed as a continuous stream, implying flow abov 25—30 ml/100 g/min. This part of the muscle flow recording is therefore omitted. The second stimulation was performed with less intensity (2 5 V) Note parallelism between muscle blood flow and aortic blood flow

Results

1 *Defence area stimulation* Fig 1 shows the effect of hypothalamic defence area stimulation in a dog with intact vagi and carotid baroreceptors but with skeletal muscles paralyzed with gallamine Aortic blood flow was here continuously recorded by means of the electromagnetic flow meter At the onset of the stimulation there was a characteristic transient decrease in aortic flow lasting less than 10 seconds, followed by a 70 per cent flow increase beyond the pre-stimulatory level Other experiments showed that this initial decrease in aortic blood flow which wa seen in the great majority of the experiments, coincided with the prompt onset of asoconstriction in the kidneys and the gastrointestinal tract In this experiment this decrease in aortic flow also coincided with a prompt but slight increase in central venous pressure as seen from the figure Calculation of the total peripheral re sistance during this initial phase of decreased aortic flow reveals doubled resisti load for the left ventricle in other experiments this nitial effect could imply a much as threefold increase in the resistive load on the left ent le

In this as in most cases, the muscle blood flow increase was delayed some 10 sec as compared with the almost immediate onset of renal and intestinal asoconstriction recorded in other experiments The m scle flow ncre w ncided w th the shift from the decreased to the increased aortic blood flow and t reached ts peak when this flow increase became maximal In subsequent t m lations, the muscle blood flow increase could be markedly reduced b, ng ne It is f rther

seen from Fig 1 that after 30—40 sec of continuous hypothalamic stimulation the muscle blood flow had returned towards the initial value. This was also a fairly characteristic feature, when hypothalamic stimulations were maintained for long periods. On the other hand the effects on heart rate and blood pressure, for example, are generally well sustained.

In some experiments, a high initial level of muscle blood flow was seen indicating a low or insignificant vasoconstrictor fibre tone In these instances the onset of vasodilation was almost immediate, and then the initial, phasic decrease in the aortic blood flow was absent, the aortic flow increasing from the very start of the stimulation. This will be discussed below

With respect to the thermodilution determinations of C. O., performed before during and after defence area stimulations in "curarized" animals but before any β-adrenergic blocking drug or atropine had been given, the results were here pooled together Under these experimental conditions, the average control value for C. O was close to 500 ml/min for a mean body weight of 3.0 kg Defence area stimulation produced maximally a twofold rise in C O during the period of 10—40 sec after the start of stimulation, with a less extensive increase during the subsequent 45—75 sec after the onset. As has been pointed out, with the thermodilution method it was not possible to reveal the transient C O decrease, occurring during the first 10 sec of stimulation.

Fig 2 shows diagrammatically the pooled data from 9 expts in "curarized" cats where the defence area stimulations were repeatedly induced before and after administration of the β-adrenergic blocking agent, H 56 28. The only such experiments included were those where the increase in muscle blood flow was more than 3 times upon hypothalamic stimulation, and was predominantly due to the cholinergic vasodilator fibres as revealed by subsequent administration of atropine. The vagi had been cut in these experiments but at least one of the carotid baroreceptor stations had been left intact Results in other experiments suggest, however that the cardiovascular response pattern was largely the same whether or not the vagal nerves were intact, although when they were […] an often pronounced poststimulatory bradycardia was seen. Whether or not the adrenal medulla was intact appeared to be of little relevance for the extent of this autonomically induced rise in C. O but the blood-borne catecholamines may help to prolong the response to some extent.

It is seen from Fig 2 that defence area stimulation increased muscle blood flow an average of 350 per cent before the β-adrenergic blocking agent was administered. This flow increase was partly a consequence of the blood pressure increase but was mainly due to a vasodilation that involved both an inhibition of constrictor fibre tone and, especially an activation of the cholinergic dilator fibres It is further obvious from this diagrammatic illustration that, in agreement with the results shown in Fig 1 the increase in the muscle blood flow was only transient a phenomenon that will be commented upon below Well correlated in time to the establishment of the muscle vasodilation, there was a rapid increase in C O preceded by a transient decrease as revealed by the electromagnetic flow meter The maximum of

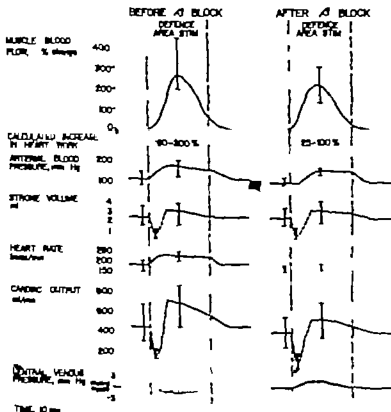


Fig. 2. Effects of defence area stimulation pooled data from 6 thermodilution experiments in cats before and after administration of β-adrenergic blocking agent (H 56/28). The heavy lines shows the arithmetic means. Total spread of values is indicated. The indicated initial decrease in aortic flow and stroke volume was calculated from 4 other experiments, where the electromagnetic flow meter was used, and these values are connected with the rest of the curves with hatched lines. Total spread of values in CVP is indicated by shaded area. For further explanation see text.

C O increase was reached within 20—30 sec Both stroke volume and heart rate rose swiftly Central venous pressure (CVP) here indicated only as changes from the resting equilibrium, could shift in both directions, but these shifts were very modest and, independent of their direction, there was always an increase in stroke volume.

Administration of 1 mg kg of the β-adrenergic blocking agent (H 56,28) did not change appreciably the resting equilibrium, presumably owing to the weak β-adrenergic stimulating action of this agent. Thus, no rise in resting CVP was observed after administration of the β-adrenergic blocker However the character of the response pattern to defence area stimulation was in some respects considerably changed As expected, the stimulation no longer produced any significant heart rate increase in these vagotomized animals, their pulse frequency now being

stabilized around 1 0 beat min. On th other hand, C. O still increased somewhat upon stimulation but much less so than before the β-adrenergic blockade. This C.O increase was thus only due to a raised stroke volume which, in turn, should be related to the now more considerable and regular rise in CVP in connection with the hypothalamic stimulation. Evidently this stroke volume increase was a consequence of the Frank Starling relationship. There was no really significant decrease in the extent of muscle vasodilation after β-receptor blockade, but total peripheral resistance showed a tendency to reach a slightly higher value during the stimulation.

If atropine was given instead of the β-adrenergic blocking agent the result was an unchanged or even decreased cardiac output upon defence area stimulation. In this situation the blood pressure rise was usually profound. As a result the stroke volume now went down despite an enhanced sympathetic discharge to the heart as indicated by the fact that the centrally induced, increased positive chronotropic action on the heart persisted. Similar results could be obtained if instead of administering atropine the position of the hypothalamic electrode was shifted to an area from which a generalized sympathetic activation, without participation of cholinergic dilator fibres, was induced (hypothalamic "constrictor" response; see e.g. Feigl 1964

In several experiments defence area stimulations were carried out before gallamine was given. The resulting changes in C. O etc., were then of essentially the same magnitude as when full muscle paralysis had been induced. It should be stressed, however that general anesthesia tends to suppress more or less completely the often ... skeletal muscle activation normally visible when defence area stimulation is performed in unanesthetized animals. In such a situation an element of ... hyperemia must be superimposed on the cholinergic vasodilator fibre effect, and therefore the C. O increase may be expected to be even greater than in the present experiments

2. *Depressor area stimulation* In all the experiments to be discussed in this section the animal were curarized by administration of gallamine and artificially ventilated to avoid stimulus-induced respiratory or other somatomotor changes that might secondarily affect the primary cardiovascular response to depressor area stimulation. Further in all the experiments the animals were vagotomized, either from the start or in the course of the experiment and often the carotid arteries were occluded in order to create a high initial level of sympathetic activity

Fig 3 shows the effect of an intense depressor area stimulation in one experiment where the tonic sympathetic activity was initially high, since the common carotid arteries were occluded and the vagal nerves cut. When the stimulation was started, the arterial blood pressure fell rather precipitously during the first phase of the stimulation and simultaneously both muscle blood flow and C. O. showed a transient increase. It should incidentally be stressed that this transient increase in muscle blood flow which occurred despite the fall in perfusing pressure was eventually unchanged when atropine was given in amounts that completely block the

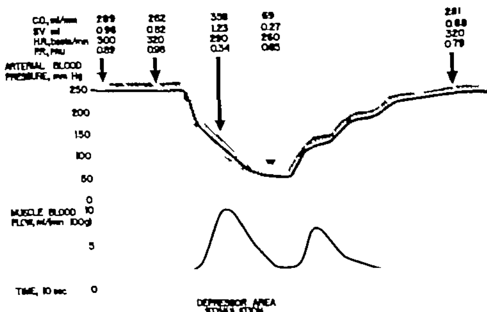


Fig 3 Cat 3 0 kg Stimulation in the medullary depressor area. Shaded area indicates relativ changes in pulse amplitude. On top of figure are calculated cardiac output from the thermodilution method, computed stroke olume heart rate and estimated total peripheral resistance For further explanation see text.

cholinergic vasodilator fibres. Also heart rate and total peripheral resistance were red ced during this early phase of the depressor response All these events must be essentially ascribed to a generalized inhibition of sympathetic tonic activ ty the result of which became most striking with respect to the muscle blood flow resistance, because sympathetic constrictor fibre ctivity is most intense in this ascular bed to start with in the ctual experimental situation (cf Löf ing 1961)

The hypothalamic sympatho-inhibitory area which seems to induce its effects ia the medullary depressor area (cf Folkow Johansson and Öberg 1959 Löf ing 1961) was timulated instead in a few experiments where t proved possible to localize it exactly Apart from the fact that the changes thus induced were less pronounced than those from the medullary depressor area, they were, as expected, of essentially the same character

Fig 4 summarizes diagrammatically the changes in most parameters studied in these experiments. This diagram is based only on such experiments where blood pressure fell from an initial level above 180 mm Hg t w ll below 100 mm Hg upon depressor area stimulation. The direction of the responses was ultimately the same in all these depressor area stimulations, though the initial phasic increase in C O entirely dependent on a temporarily increased stroke ol me, since heart rate decreased — could be clearly traced only in about half of the experiments How ever muscle blood flow resistance remained reduced throughout the stimulation period. The diagram lso show that the cardiovascular depressor response generally

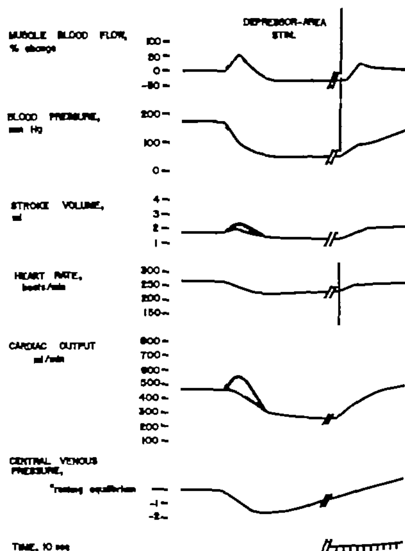


Fig. 4 Effects of medullary depressor area stimulation pooled data from 6 expts. in cats, the heavy lines indicating the arithmetic means. The individual variations of the initial rise in cardiac output and computed strok olume are indicated by the shaded area. For further explanation see text.

reached its nadir only after some minutes of continued stimulation when cardiovascular collapse was often imminent

The C. O could sometimes be further decreased by tilting the animal with the hindlegs down, presumably because this increased the extent of venous pooling in dependent parts of the vascular bed. However since the fraction of the blood volume caudad to the heart is not as great in the cat as it is in man, the pooling effect induced by this manoeuvre wa not so striking A more powerful influence in this respect was exerted in these curarized" animals by the positive pressure breath-

ing, which leads to more generalized pooling of blood outside the thorax. Also previous bleeding of the animal was found to intensify the decrease in C O and blood pressure, caused by depressor area stimulation. All these manoeuvres in an otherwise intact animal would also tend to somewhat reduce venous return, and would then normally be compensated for by reflexly increased sympathetic activity Hence, the depressor area stimulations, by inhibiting this activity produce more drastic reductions in both pressure and C. O and more profound venous pooling thus simulating the events seen during fainting in upright man.

Discussion

In the present study two centrally elicited cardiovascular adjustments, namely the hypothalamic defence reaction and the medullary depressor response, have been compared with special regard to changes in cardiac output (C. O) and central hemodynamics. While these two responses are diametrically opposite in practically all respects, they both induce the same vascular *eff ct r* response in the skeletal muscles, as evidenced by a decreased flow resistance in this tissue. This finding may give the impression that the same neurogenic mechanisms are involved in producing the skeletal muscle vasodilation in both instances. This aspect will be discussed first.

In the defence reaction there is little doubt that, at least in cats and dogs, the muscle vasodilation is mainly due to an activation of cholinergic dilator fibres. Initially however the vasodilation appears as a result of reflex inhibition of con strictor fibre tone via baroreceptor stimulation, when the arterial blood pressure and pulse amplitude begin to rise. The active cholinergic vasodilation is usually characterized by a slightly delayed, but marked secondary increase in muscle blood flow (*e.g.* Feigl, Johansson and Löfving 1964) The delayed onset of "active vasodilation may be the result of a differentiated neuro-effector organization in the muscle resistance vessels (Folkow Öberg and Rubinstein 1964) The full impact of the cholinergic vasodilation in the skeletal muscle becomes clearly evident first when the vasoconstrictor fibre activity is reflexly inhibited. In a few experiments in which the control blood flow rates were high in muscle, indicating a negligible vasoconstrictor tone the element of initial reflex inhibition was consequently absent and the activ cholinergic vasodilation appeared immediately upon the stimulation of the defence area.

It is further interesting to not that the cholinergic vasodilation is usually not sustained during prolonged defence area stimulation. The centrally induced vasodilation is in most cases promptly counteracted by local autoregulatory mechanisms the muscle blood flow increases beyond the ctual tissue requirements. This inter esting type of escape phenomenon has been analysed in another publication (Djojosugito t al. 1967)

On the other hand, the muscle vasodilation seen in the centrally induced de pressor response assumes entirely different characteristics. There is an initial, some

times rather marked increase of muscle blood flow despite a concomitant decrease in arterial pressure, followed by a decrease in muscle blood flow to or below control level as the blood pressure decline proceeds (see Fig 3 and 4) The initial increase in muscle blood flow may be so marked as to surpass that seen in an acutely sympathectomized limb apparently suggesting an element of "active" vasodilation. Such an observed "over-shoot" in muscle blood flow during human fainting led to the suggestion that specific vasodilator fibres might be involved in this centrally mediated depressor response in man (Barcroft and Edholm 1945) However the present experiments on cats showed that this phasic muscle blood flow increase induced by depressor area stimulation, does not involve any cholinergic dilator fibre activation, since the response was the same after complete atropinization (see also Löfving 1961) Recently other types of active neurogenic vasodilator mechanisms have been suggested (Beck 1961 Abboud and Ballard 1967 Brody 1967 and Tuttle 1967) but it may still be too early to assess for definitely this interesting possibility. Quite apart from such a possibility it is not surprising that a brief over-shoot in muscle blood flow can occur simply as a result of a sudden inhibition of vasoconstrictor fibre activity After all, such an activity implies a flow restriction for the tissue and its sudden elimination should consequently result in a brief reactive hyperemia analogous to that seen in an acutely sympathectomized limb. Such a phasic flow increase has also been repeatedly observed in this laboratory upon sudden interruption of a direct vasoconstrictor fibre stimulation to the muscle vascular bed. Thus the mechanism of skeletal muscle vasodilation associated with the studied depressor response may after all be solely the result of inhibition of energic contractor fibre activity in any case it differs distinctly from the cholinergic vasodilation observed in the defence response

In essentially all other respects, these two centrally induced cardiovascular response patterns are diametrically opposite Thus can be easily visualized as the defence reaction involves an increase in sympathetic cholinergic as well as adrenergic discharge, coupled with reflex inhibition of vagal impulses to the heart, while the depressor response on the other hand, results in a generalized inhibition of sympathetic discharge combined with a reflex increase of vagal tone Thus the defence area activation results in an increase in arterial pressure and tachycardia and the depressor area stimulation leads to a decrease in arterial pressure and bradycardia. Consequently the changes in C O in these two responses are also like mirror images. With defence area stimulation a brief initial decrease in C O is seen in the majority of experiments, after which C O rapidly increases up to twofold above control level, under the present experimental conditions. On the other hand, with depressor area stimulation a transient and slight increase in C. O is often first observed in most cases followed by a profound decrease The decrease can be further accentuated if the transmural pressure in the capacitance side of the vascular bed is somewhat raised by tilting or positive pressure breathing to favour peripheral pooling of blood upon sympathetic inhibition, or if the animal has been exposed to a blood loss prior to the stimulation.

The brief decrease in C. O at the onset of defence area stimulation could be explained by a transitory rise in arterial pressure arising from the sudden increase of resistance in the vascular beds of the skin, the kidney and the intestines. Despite the concomitant neurogenic adjustment of the heart to increase rate and contractile force, the left ventricle can then not promptly match this sudden increase in flow resistance, partly because venous blood mobilization is usually somewhat delayed, as compared with the resistance increase. The subsequent C. O increase is closely related to the usually slightly delayed skeletal muscle vasodilation. The latter must be the most significant, though not the sole, factor responsible for the elevation in C. O for instance, coronary blood flow can also be expected to increase. The delay in the onset of C. O increase always coincided with the delayed onset of muscle flow increase. In those experiments in which muscle flow increase was immediate because of an initially insignificant vasoconstrictor fibre activity C. O increase was also immediate.

With prolonged defence area stimulation, tachycardia was well sustained but skeletal muscle vasodilation was not due to local autoregulatory adjustments (Djojosugito *et al.* 1967) C. O after reaching a peak, then also declined parallel to the decrease in muscle blood flow On the whole, in these curarized animals it was usually impossible to impose upon the muscle vascular bed a neurogenic flow increase for any length of time, because of this autoregulatory return of muscle vascular tone towards control, flow being then again balanced to the unchanged metabolic demands of the curarized muscles. It should be pointed out, however that in the unanesthetized animal, defence area activation is also associated with more or less extensive skeletal muscle activation, in which case an element of exercise hyperemia must be superimposed on the cholinergic vasodilating effect to further increase the C. O Thus, in the unanesthetized state the increase in C. O to defence area activation is likely to be more than twofold, the maximum recorded under the present experimental condition, and it is probably also far more sustained. The present experiments were, however designed to reveal only the strictly autonomic adjustments of C. O and peripheral flow

In the case of the depressor response elicited either from stimulation of the medullary depressor area or from certain cortico-hypothalamic structures (*cf* Löfving 1961) C. O could increase to some extent initiall but then C O fell considerably below control. The initial increase reflects a sudden fall in flow resistance which appears to precede slightly the full impact of sympathetic inhibition on the veins and central blood depots in the lungs and also on the heart. For a short period, the left ventricle would then expel slightly increased volume and hence increased output, before the reduction in venous return and myocardial contractility is fully established. Following this transient increase in the C O sometimes profound fall can occur If venous pooling is further exaggerated by tilting or positive pressure breathing, the C. O can, indeed, be reduced to such an extent as to cause an imminent cardiovascular collapse

Both the defence and depressor responses are known to occur in man. The

defence response is seen primarily with emotional distress. While the increase in heart rate is usually only moderate during defence area activation in the anesthetized animal emotional stimuli often lead to profund tachycardia and blood pressure rise in man resulting in considerable increase in work load of the heart (*cf* Brod 1961) It is interesting to note, from the present experiments, that the increase in cardiac work in response to defence area stimulation, which could be threefold, could be reduced by 50—60 per cent after administration of a β-adrenergic blocking compound. This reduction in the acute strain on the heart resulting from pharmacological blockade of cardiac sympathetic drive might be of relevance in man when a reduction of strain on the heart due to emotional stress is desirable After all, an emotionally induced threefold increase in the work load on the heart corresponds to the load imposed by fairly extensive muscular exercise

Marked depressor responses in man are seen in *e.g.* emotional fainting Since man is normally in the erect position profound venous pooling in the dependent part of the body may occur together with hypotension. This may indeed lead to cardiovascular collapse, which would hardly occur if the same centrally induced depressor response were induced in a subject in the supine position. However these episodes of emotional sympathetic inhibition are usually very short further they are accompanied by suppression of prevailing somatomotor activity Therefore the individual inevitably would assume a recumbent position which in turn helps to restore venous return by lowering the transmural pressure within the capacitance vessels, thus greatly reducing the hemodynamic impact of the centrally induced depressor response

This study was supported by grants from Air Force School of Aerospace Medicine under contract AF 61(052) 732 through the European Office of Aerospace Research (OAR) United States Air Force from U.S. Public Health Service (HE-05675-06), from the Swedish Medical Research Council (B67 14X 16-03B) and from the Faculty of Medicine University of Göteborg
AB Hässle Göteborg Sweden, has generously covered the employment of technician and supplied the β-blocking drug H 56/28.

References

Ablad, B M Brogård and L Ek Pharmacological properties of H 56/28—a β-adrenergic receptor antagonist *Act pharmacol* (Kbh.) 1967 *25* Suppl. 2.
Abboud, F M and D R Bullard Release of vasoactive substances from forelimb and kidney during nerve stimulation *Fed Proc* 1967 *26* 715
Abrahams, V C S. M Hilton and A Zbrożyna Active muscle vasodilatation produced by stimulation of the brain stem Its significance in the defence reaction. *J Physiol.* (Lond) 1960. *154* 491 513
Barcroft H and O G Edholm On the vasodilatation in human skeletal muscle during posthaemorrhagic fainting *J Physiol.* (Lond) 1945 *104* 161—175
Beck, L Active reflex dilatation in the innervated perfused hindlimb of the dog. *Amer J Physiol.* 1961 *201* 123—128
Brod, J *Haemodynamic* [illegible] *on the haemodynamic basis of essential hypertension.* Proc of Joint WHO-Czechoslovak Cardiological Society Symposium on the Pathogenesis of Ess Hypertension. Stat Medical Publ. House Prague 1961 256—264
Brody M. J Distribution of vasodilator nerves in the canine hindlimb. *Fed Proc* 1967 *26* 550

Djojosugito, A. M., B. Folkow, B. Lisander and H. Sparks, Mechanism of Escape of Skeletal Muscle Resistance Vessels from the Influence of Sympathetic Cholinergic Vasodilator Fibre Activity. *Acta physiol. scand.* 1968. 72. 148—156.

Fegler, G., Measurement of cardiac output in anesthetized animals by the thermodilution method. *Quart. J. exp. Physiol.* 1954. 39. 153—164.

Feigl, E. O., Vasoconstriction resulting from diencephalic stimulation. *Acta physiol. scand.* 1964. 60. 372—380.

Feigl, E. O., B. Johansson and B. Löfving, Renal vasoconstriction and the defence reaction. *Acta physiol. scand.* 1964. 62. 429—435.

Folkow, B., C. Heymans and E. Neil, *Integrated aspects of cardiovascular regulation.* Handbook of Physiology. Sec. 2. Vol. 3. 1787—1823.

Folkow, B., B. Öberg and E. H. Rubinstein, A proposed differentiated neuro-effector organization in muscle resistance vessels. *Angiologica.* 1964. 1. 197—208.

Folkow, B., B. Lisander and S. C. Wang, Changes in cardiac output in cats during stimulation of the hypothalamic defence area and the bulbar depressor area. *Acta physiol. scand.* 1966. 68. Suppl. 277. 50.

Folkow, B., P. Hedner, B. Lisander and E. H. Rubinstein, Release of cortisol upon stimulation of the hypothalamic defence area in cats. A symposium on stress. *Försvarsmed. Tidskrift* 1967. In press.

Johansson, B., Circulatory responses to stimulation of somatic afferents. *Acta physiol. scand.* 1962. 57. Suppl. 198.

Korner, P. I., The effect of section of the carotid sinus and aortic nerves on the cardiac output of the rabbit. *J. Physiol. (Lond.)* 1965. 180. 266—278.

Löfving, B., Cardiovascular adjustments induced from the rostral cingulate gyrus. *Acta physiol. scand.* 1961. 53. Suppl. 184. 1—82.

Rosén, A., Augmented cardiac contraction, heart acceleration and skeletal muscle vasodilatation produced by hypothalamic stimulation in cats. *Acta physiol. scand.* 1961. 52. 291—308.

Rushmer, R., *Effects of nerve stimulation and hormones on the heart: the role of the heart in general circulatory regulation.* Handbook of Physiology. Sec. 2. Vol. 1. 533—550.

Tuttle, R. S., Evoked and reflex release of histamine in the pyramidal cat. *Fed. Proc.* 1967. 26. 2561.

Uvnäs, B., *Central cardiovascular control.* Handbook of Physiology. Sec. 1. Vol. 2. 1131—1162.

Acta physiol. scand. 1968. 72. 234—247

From the Institute of Neurophysiology University of Oslo, Norway

Nervous Control of Urinary Bladder in Cats

IV Effects of autonomic blocking agents on responses to peripheral nerve stimulation

By

PER EDVARDSEN

Received 23 June 1967

Abstract

EDVARDSEN P *Nervous control of urinary bladder in cats IV Effects of autonomic blocking agents on responses to peripheral nerve stimulation.* Acta physiol. scand. 1968. 72 234—247

The bladder response to electrical stimulation of the parasympathetic and sympathetic nerves was recorded in anesthetized cats Stimulation of the pelvic nerves caused sustained bladder contraction. This response was not changed significantly by blockade with *atropine* at 5 cps stimulation, while at 30 cps onl an initial contraction was obtained. It was abolished *meth* um whereas t remained unchanged after *guanethidine* *propranolol* and *dibenzy-* There was no evidence to suggest the presence of inhibitory fibres. Stimulation of the nerves gav rise to an initial contraction followed by relaxation. On continuous ulation the slope of the pressure/v lume curv was flattened in proportion to the strength stimulation. However during the relaxation phase of the detrusor muscle, the bladder outlet responded by con raction. The sympathetic response was blocked by *guanethidi* but this, in small doses, ugmented the contraction phase. *Propranolol* increased the contraction phase and in addition converted the relaxation to contraction. The contraction response could be blocked selectively by *administration of d benzyline It is suggested that adrenergic alpha-re-*ceptors re presen both at the bladder outlet, and in the detrusor muscle as well but that in the latter ase thei presence is masked by the inhibitory beta-receptors.

In a previous communication the effects of some sympatholytic and parasympatholytic drugs on motility of the intact bladder were reported (Edvardsen 1967c) These indicated that, in agreement with previous transection experiments (Edvardsen 1967a, b) the sympathetic nerves exert an inhibitory influence on the bladder during its collecting phase while the parasympathetic nerves come into play during the micturition contraction onl Moreover after th administration of propranolol, the effects on the bladder of intraspinal operati procedures suggested that the detrusor muscle contains an adrenergic alpha receptor in addition to the beta receptor which is responsible for the inhibition normally observed after such procedures.

The aim of the present experiments was first to determine whether the parasympathetic nerves contain inh bitory fibres, as suggested by some workers (for ref see

Edvardsen 1967c) and whether the bi-phasic contraction-relaxation response of the bladder to sympathetic nerve stimulation may be ascribed to the activity of two different fibre types. Secondly it was intended to ascertain whether peripheral nerve stimulation after the administration of drugs which block either of the two divisions of the utonomic nervous system in the intact animal, revealed if sympathetic fibres were admixed with the parasympathetic nerves or *vice versa*. Finally the experiments attempted to determine the areas of distribution of the two types of adrenergic receptors in the bladder wall.

Material and methods

Twenty-six cats of either sex weighing between 1.6 and 5.5 kg were used. I 6 expts. chloralose-urethane was given intravenously in doses of 40 and 80 mg/kg respectively after induction with ether. I 20 expts. pentobarbital sodium (Nembutal® Abbott) was given intraperitoneally in doses of 30 mg/kg supplemented subsequently by small intramuscular injections when necessary. The femoral ein was used for injection of the drugs.

Isometric bladder recordings (20 expts.) were obtained after insertion of cannula into the apex of the bladder or of an indwelling urethral catheter as described in previous articles (Edvardsen 1967a, b). The former route was employed for investigation of micturition. Isotonic records (6 expts.) were obtained kymographically on smoked drum.

The hypogastric nerves were sectioned caudal to the inferior mesenteric ganglia, and the distal ends collected into one tubular stimulation electrode with a distance between the silver rings of 5 mm. The pelvic nerves wer sectioned proximal to their ramification. Each of the distal ends was isolated in stimulation electrode with distance of 3 mm between the silver rings. The latter electrodes were connected in parallel for the simultaneous stimulation of both nerves. Using switch on the operating table the stimuli could be directed towards either the hypogastric or the pelvic nerves, the other pair serving as control to the preparation.

Square wave pulses were delivered through stimulus isolation unit. When the effects of drugs were tested, the stimuli parameters were kept constant 1 ms 5 and 30 cps for 15 sec for period pproximately corresponding to that of spontaneous micturition contraction.

Results

(1) Bladd ponses t peripheral erve stimulatio

( ) *St m lat on f th parasympathetic nerves* (6 expts.) The pelvic nerves were stimulated nder isometric (3 expts.) and isot nic conditions (3 expts.) In 4 experiments the stimulation was preceded by section of the hypogastric nerves, while in the other two the sympathetic nerv supply was intact. There were no essential differences the responses of the bladder in th presence or absence f sympathetic innerv tion, nor had arying bladder olumes any noticeabl effect.

Stimulation t intensities just above the threshold ga rise to a contraction, ir respectiv of whether t was obtained by increasing the frequency the duration, or the ntensit of the stimul (Fig 1 A) When either of the parameters was increased the bladder response was augmented, and, t frequencies of p to 30 cps lasted for the period of stimulation. At higher frequencies there w f t qu if th timulation was maintained for more than 15—20 sec

H xam keanam w kindly supplied by M and Baker Ltd Dagenham *p propranolol* (Inderal® by Imperial Chemical Industries Ltd Manchester and *guanethidine* (Ismelin®) CIBA Société Anonyme Basel.

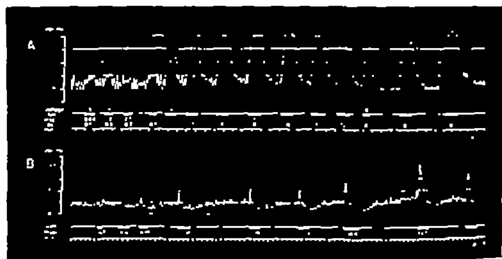


Fig 1 *A* Effects on bladder response of varying parameters of pelvic nerve stimulation. Exhaustion has occurred at 32 cps and relaxation after contraction at 8 V. No relaxation after stimulation when the bladder is empty (last record). Volumes indicated on figure. Isotonic recording, indirect cystometry. Chloralose-urethane anesthesia. *B*. Effects of hypogastric nerve stimulation 5 V at 8 cps for 2—3 min, on increasing stimulus duration (msec). Isotonic recording, indirect cystometry. Chloralose-urethane anesthesia.

After cessation of stimuli of up to a critical level of intensity the bladder resumed its resting volume or pressure, depending on the recording. A further increase in the stimulus intensity was followed by relaxation after the contraction. This relaxation was not seen if the bladder was stimulated at identical intensities while empty and then allowed to fill (Fig. 1 A, last stimulation). The relaxation was enhanced, on the other hand, if the bladder was allowed to contract isometrically, i.e. when the catheter was closed during the stimulation, but became less pronounced, or disappeared altogether on repeated stimulation.

(b) *Stimulation of the sympathetic nerves* (8 expts.). In this group 4 experiments were performed under isometric and 4 under isotonic conditions. The parasympathetic nerve supply was intact in four of the animals, in order to observe the effect of sympathetic nerve stimulation on the micturition contraction, while in four experiments the stimulation was performed after parasympathetic neurotomy. The bladder response were similar in both groups, suggesting that the presence or absence of the parasympathetic nerve supply was immaterial. They were not influenced essentially by the type of anesthesia used or by the recording technique, although the relaxing effects of the stimulation were more readily produced under isotonic conditions.

On intermittent stimulation of the hypogastric nerves the bladder response consisted of two phases, i.e. a rapid contraction superseded by relaxation or inhibition. Unlike the response to pelvic nerve stimulation, the contraction was never sustained. Nevertheless, more than half the bladder contents could be expelled by the contraction if the outlet was kept open by a urethral catheter. The inhibition obtained by

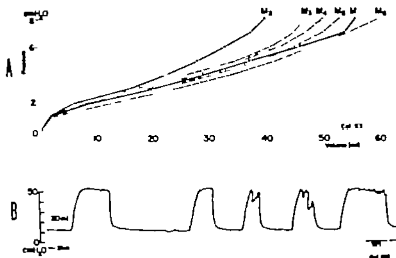


Fig. 2. *A* Effects of hypogastric nerv stimulation, 6 V 5 ms. Pelvic nerves intact. Full-drawn lines indicate bladder pressure/volume curv of intact animal (M) and after sectioning hypogastric nerves (M) broken lines ditto on hypogastric nerve stimulation at 1 cps (M$_3$) 2 cps (M) 4 cps (M$_5$) and 8 cps (M) Micturition reflex licited t identical levels (8 cm H_2O) Isotonic recording, indirect cystometry Chloralose-urethane anesthesia. *B*. Effect of hypogastric nerv stimulation, 4 V 5 cps, 1 ms on recurrent micturition contractions (M) Bladder volume 20 ml. Isometric recording, indirect cystometry Pentobarbital anesthesia.

further stimulation led to the disappearance of the spontaneous contractions. When the stimulation was interrupted a further sudden relaxation occurred, followed by a gradual return in the course of a few minutes to the pre-stimulatory volume or pressure (Fig. 1 B) At the same time the spontaneous contractions reappeared. The recovery time depended upon the intensity and duration of the stimulation, being prolonged in proportion to the intensity

At threshold intensities it was found that if a contraction response was obtained, the relaxation phase always followed (Fig. 1 B) A primary relaxation or an isolated contraction never occurred. On ncreasing the stimulus intensity both the initial bladder contraction and the relaxation phase became more pronounced. The same effects on both phases were obtained when the bladder olume was increased, although t large bladder volumes the relaxation phase tended to predominate. Relaxation alway followed repeated stimulation.

With continuous hypogastric nerve stimulation the inhibitory effect persisted whereas the contraction response was obtained only initially It was shown on indirect cy tometry that the greater the stimulus intensity the flatter the slope of the pressure ol me curve became and, correspondingly the greater the rise in the micturition olume threshold. The stimulation did not, however alter the level of intravesical pressure at which the emptying contractions were evoked (Fig 2 A)

Stimulation of the hypogastric nerves during a spontaneous micturition contraction was performed on several occasions under both isotonic and isometric c f

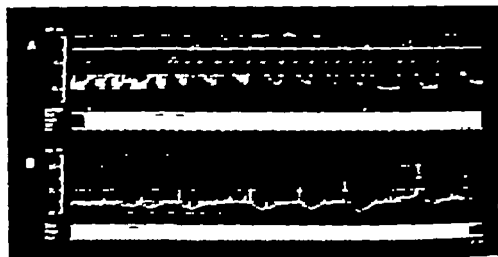


Fig. 1. *A*. Effects on bladder response of [illegible] parameters of pelvic nerve stimulation. [illegible] has occurred at [illegible] and relaxation after contraction at 8 V. No response after stimulation when the bladder is empty [illegible] record. [illegible] indicated on figure. Lower recording: indirect cystometry. Chloralose-urethane anesthesia. *B*. Effects of hypogastric nerve stimulation 5 V at 8 c/s for [illegible]–3 min, on [illegible] stimulus [illegible]. Lower recording: indirect cystometry. Chloralose-urethane anesthesia.

A[illegible] stimulation [illegible] up to a critical level of intensity, the bladder responded [illegible] [illegible] volume or pressure depending on the recording. A further increase in the stimulus strength was followed by relaxation after the contraction. This relaxation was not seen if the bladder was stimulated at identical intensities while empty and [illegible] allowed to fill (Fig. 1A, last stimulation). The relaxation was enhanced on the other hand if the bladder was allowed to contract isometrically, [illegible] when the catheter was closed during the stimulation, but became less pronounced or disappeared altogether on repeated stimulation.

b. *Stimulation of the [illegible]* [illegible] *experiments.* In this group 4 experiments were performed under [illegible] and 4 under [illegible] conditions. The parasympathetic nerve supply was intact in [illegible] of the animals in order to observe the effect of sympathetic nerve stimulation on the micturition contraction, while in four experiments the stimulation was performed after parasympathetic nerve [illegible]. The bladder response were similar in both groups, [illegible] that the presence or absence of the parasympathetic nerve [illegible] was immaterial. They were not influenced essentially by the type of anesthesia used or by the recording technique, although the relaxing effects of the stimulation were more readily produced under [illegible] conditions.

On intermittent stimulation of the hypogastric nerves the bladder response consisted of two phases, i.e., a rapid contraction superseded by relaxation or inhibition. Unlike the response to pelvic nerve stimulation the contraction was never sustained. Nevertheless, more than half the bladder content [illegible] could be expelled by the contraction if the outlet was kept open by a urethral catheter. The inhibition obtained by

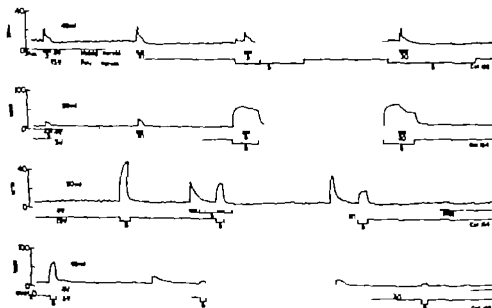


Fig. 3. Effects of simultaneous stimulation of hypogastric and pelvic nerves, stimulus duration 1 ms Intensity (V) and frequency (cps) indicated on figure Sympathetic stimulus during continuous parasympathetic stimulus (A, B) and vic ersa (C, D) Bladder olumes indicated on figure Note different scales of pressure. Isometric recording, indirect cystometry Cat 153 decerebrated, cat 152 and 154 under pentobarbital anesthesia.

The relaxation following stimulation of submaximal or maximal intensity is apparently caused by some factor in the contractil elements themsel es, since it is not obtained after stimulation of the empty bladder Whether this phenomenon is due to activation of intramural cholinergic inhibitory mechanisms similar to those postulated as being responsible for the inhibition of taenia coli (B rnstock, Campbell and Rand 1966) or to biomechanical 'sliding effects of the contraction, remains to be investig ted.

As regards the bladder esponse to *sympathetic* nerve stimulation, it ppears from these experiments that the initial motor response is evoked in a considerable part of the detrusor muscle, since as much as one-half of the bladder content or even more was expelled when indirect cystometry was employed. The lack of fluid emission on direct cystometry on the other hand, indicates that the bladder outlet also ontracts. In disagreement with Sigg and Sigg (1964) who experimented using electromy ography t is therefore suggested that both the detrusor muscle and the bladder base take part in the sympathetic motor response.

The relaxation and disappearance of spontaneous contractions during continuous mpathetic nerve stimulation indicate inhibition of the detrusor muscle The additional relaxation observed on cessation of the timulation, however suggests that even while the detrusor muscle is relaxing, the bladder base remains contracted Thus the maximum fall in pressure occurs when this part of the bladder also relaxes on ac count of interrupting the stimulus.

These experiments did not indicate that d fferent nerve fibres transmit the impulses giving rise t the excitation and to the inhibition of the bladder. It seems justified to a ume therefore that the different responses are produced at the effector level.

On *simulta cous stimulation* of th two divisions of the autonomic nerves to the bladder no evidence was found to suggest that synergism occurs. On the other hand, the sympathetic inhibition of the bladder resp nse to para ympathetic nerve stimulation does not signify that their effects are mutually antagonistic since the sympathetic response was not reduced by parasympathetic nerve stimulation. Whether the sympathetic inhibition is exerted exclusively on the effector cell or also on the parasympathetic ganglia could not be shown by these experiments.

(2) The i fl nce of som drug on th bladd r espon e

The effects on respiration, blood pressure etc of the drugs employed have been described in an earlier paper (Ed ardsen 196/c)

(a) *At opine* 6 expts.) Stimulation was not undertaken until at least 15 min had elapsed after administration of the drug

The para ympathetic bladder contraction after administration of 0.01 mg kg of atropine wa almost always reduced, the intravesical pressure being lowered by some 5—10 per cent This reduction wa no greater if the dose was increased to 0.5 mg kg. The bladder response after the administration of atropine however was seen to depend pon the stimulation frequency Thus, whereas at 5 cps there was no difference in the records btained, apart from the slight overall reduction in amplitude, at 30 cps the initial pressure peak nly reached the control level, and the pressure en fell grad ally in spite of further stimulation (Fig 4 A)

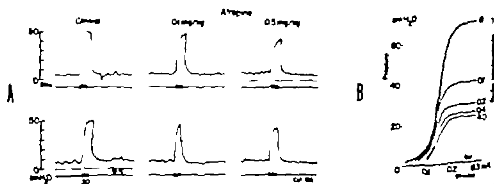


Fig. 4. *A.* Effect of atropine on bladder response to pelvic nerve stimulation 5 V 1 ms at 5 and 30 cps. Atropine doses indicated t top. Bladder olume 20 ml. Isometric recording. Indirect cystometry. Pentobarbital anesthesia. *B.* Effect of hexamethonium on bladder response to pelvic nerv stimulation 5 cps, 1 ms after atropine 0.2 mg/kg. Stimulus intensity indicated on abscissa, bladder pressure on ordinate hexamethonium dose to the right. Bladder volume 40 ml. Isometric recording, ind rect cystometry Pentobarbital anesthesia.

The contraction response to sympathetic nerve stimulation was less constantly reduced by atropine in these doses, and no difference in the patterns of response was seen. The relaxation phase of the sympathetic bladder response was apparently uninfluenced by this drug.

(b) *Hexamethonium* (4 expts.) The bromide salt was employed, and stimulation was not started until at least 30 min after its administration.

The parasympathetic bladder response was reduced, as indicated by a lowering of the intravesical pressure. The effect was marked after doses of 0.1 mg/kg, and was nearly doubled after 1 mg/kg. A further increase in the dose produced a less marked change (Fig. 4 B). After 4 mg/kg no f rther diminution in the response was obtained. The effects were essentially similar at frequencies of 5 and 30 cps, although the residual response was greater at the higher frequencies.

The sympathetic bladder response was not changed significantly by hexamethonium, which indicates that most of the fibres affected by the stimulation were postganglionic.

Atropine did not alter these effects of hexamethonium.

(c) *G anethidi* (5 expts.) Guanethidine was administered in doses of between 0.01 and 10 mg/kg body weight. Stimulation was not started until 30 min after the injection.

The parasympathetic bladder response was not affected by the drug when given in doses of up to 10 mg/kg at any of the stimulation frequencies. In particular guanethidine had no effect on th relaxation following the contraction.

The response to sympathetic nerv stimulation, on the other hand, was markedly affected. At doses of 0.01 and 0.02 mg/kg the bladder contraction response was enhanced, while it was diminished when the dose exceeded 0.05 mg/kg (Fig. 5 A) *i.e.* that amount which affects the systemic blood pressure (Ed ardsen 1967c). A submaximal blockade of the response was obtained after doses of 0.25 mg/kg but even after 10 mg/kg a slight contraction response was still obtained.

The relaxation phase of the bladder response was related to the contraction response in similar way to that observed following threshold stimulations. *i.e.* the more the motor response was reduced by the drug th less was the subsequent relaxation. This observation was confirmed in two dditional experiments performed under isotonic conditions. But, conversely the potentiation of th small doses on the contraction response was not associated with a corresponding enhancement of th inhibition.

The subsequent administration of atropine had no further effect.

(d) *P pr olol* (5 expts.) Propranolol was given in doses of from 0.05 to 2 mg/kg body weight. At least 30 min was allowed t elapse after administration of the drug before stimulation of the nerves was started.

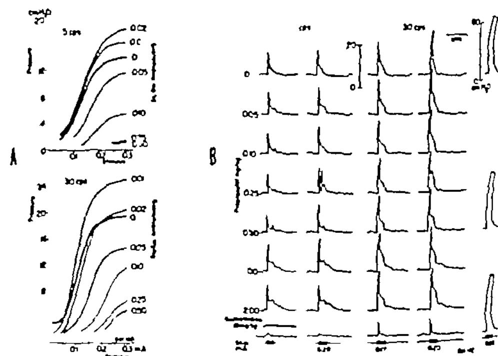


Fig. 5. *A.* Effect of guanethidine on contraction response t hypogastric nerv stimulation at 5 and 30 cps 1 ms, f er tropine 0 5 mg/kg. Stimulus intensity indicated on abscissa, bladder pres ure on ordina guanethidine dose to the right. Note excitatory effects of small doses. Bladder volume 30 ml Isometric recording indirect cystometry. Pentobarbital anesthesia. *B.* Effect of propranolol on bladder response to submaximal and maximal hypogastric nerve mulation 5 and 30 ps, 1 ms, after atropine 0 mg/kg. The pelvic nerve control response to th right. Stimulation intensit (mA) indic ted on signal t bottom, propranolol dose to the left, guanethidine administration a rrow. Bladder olume 20 ml. Isometric recording indirect cystometry. Decerebrated. Responses redr w from records.

The parasympathetic bladder response was not changed by the drug (Fig 5 B) at either of th timulation frequencies used

The sympathetic response however showed gradual change as the dose was increased in that the ontraction phase became more predominant and tended to last longer while the subsequent relaxation hanged into a contraction which became more prominent as the doses were increased up to 1 mg/kg. Above this level no further increase occurred Th responses were essentially similar at submaximal and maximal stimulation intensities and at frequencies of 5 and 30 cps (Fig 5 B)

The change was not altered by atropine Guanethidine on the other hand, completely blocked the propranolol induced contraction, and reduced the stimulatory contraction as well just as when guanethid ne alone was given (Fig 5 B)

In two add tional experiments the sympathetic nerves were cut and stimulated, leaving the micturition reflex intact. Direct cystometry showed that stimulation of the hypogastric nerves after the administration of 1 mg/kg of propranolol caused a forceful bladder contraction, but that no fluid was expelled as long as the nerves were being stimulated. Spontaneous micturition occurred, however once the stimuli were

Discussion

The results of the present experiments suggest that the parasympathetic nerves mediate impulses which evoke exclusively motor responses in the bladder. The sympathetic nerve impulses give rise to, on the other hand, an initial contraction of the whole organ supervened by relaxation of the detrusor muscle with persistent contraction of the bladder base. The bladder response to parasympathetic nerve activity is inhibited by sympathetic nerve stimulation. Synergism between the two divisions of the autonomic nerves to the bladder has not been demonstrated by the investigation.

The bladder outlet appears to constitute an internal vesical sphincter, as was suggested by Clegg (1957) from anatomical investigations, but the functional significance of this sphincter is not clear since the bladder is still continent after sympathectomy. On the other hand, a sympathetically induced contraction of the bladder base in conjunction with the relaxation of the detrusor muscle gives optimal effects of the sympathetic reflex activity during the collecting phase of the bladder (Edvardsen 1967a).

The mechanisms brought into play by the sympathetic inhibition of the bladder response to parasympathetic nerve activity are not elucidated by these experiments. The sympathetic inhibition of denervated bladders suggests that the action is on the effector cells. This may also be assumed from the sympathetic inhibition of the bladder during its collecting phase, when no background activity is present in the parasympathetic nerves. On the other hand, the sympathetic terminals on parasympathetic ganglion cells in the bladder wall described by Hamberger and Norberg (1965) would suggest sympathetic inhibition of the parasympathetic ganglion transmission as well. It thus seems likely that the sympathetic inhibition is exerted at both the effector and the parasympathetic ganglion level, thus allowing maximum efficiency of sympathetic effect both in guarding and continence reflex activity (Garry, Roberts and Todd 1959; Kuru 1965) and in the voluntary interruption of micturition (Edvardsen 1967b).

Referring to the adrenergic receptors, the alpha-receptors appear to be located mainly at the base of the bladder but seem also to be distributed in the detrusor muscle. The following observations, made on sympathetic nerve stimulation, are in favour of this latter assumption. ( ) The initial bladder contraction can expel more than half the bladder contents when the outlet is kept open by a urethral catheter. (ii) The initial pressure rise, in addition to that already evoked by maximum parasympathetic nerve stimulation, suggests that contraction mechanisms other than cholinergic are involved. (iii) An adrenergic contraction mechanism activating the detrusor muscle is suggested by the guanethidine experiments, where the potentiated motor response following small doses was obtained only on stimulation of the hypogastric nerves, while the response to pelvic nerve stimulation was unaltered. ( ) The raised bladder pressure obtained after the administration of propranolol which is higher than that of a spontaneous micturition contraction, indicates distribution

The effect of *atropine* on the parasympathetic bladder response is compatible with the theory propounded by Hucovié Rand and Vanov (1965) that there is a competitive reversal of atropine blockade by high local concentrations of acetylcholine released from the nerve terminals. The assumption of Langley (1911) that the rapid exhaustion of the bladder at stimulation after the administration of atropine was due to a decreased blood flow caused by the drug leaves unexplained the different responses obtained at the different stimulation frequencies, when the amplitude of the contractions may be identical. The dependence on stimulation frequency of the bladder response after atropine may explain the conflicting reports of the effects of the drug made by Henderson and Roepke (1935) and by Ursillo and Clark (1956). The first named authors observed the persistence of an initial contraction which decreased on further stimulation, while the latter two using a stimulation frequency of 15 cps did not observe the fatigue phenomenon, but noted an overall reduction in pressure.

The lack of effect of *hexamethonium* on the sympathetic bladder response suggests that this contraction phase is not due to the excitation of parasympathetic bladder ganglia. Moreover if there is any parasympathetic representation in the hypogastric nerves, it appears to be small and of no functional significance. The reduced rise in pressure following sympathetic nerve stimulation after the administration of tetraethylammonium chloride (TEAC) as a blocking agent reported by Root (1947) was not observed after hexamethonium blockade. According to Root TEAC produced similar effects upon pre- and postganglionic stimulation, however, and also had no effect on the muscular tone of intact bladders, unlike hexamethonium (Edvardsen 1967c). The two drugs thus seem to have different modes of action.

The unchanged parasympathetic bladder response after the administration of *guanethidine* suggests that any sympathetic representation that may be present in the pelvic nerves is of little functional significance, although the hexamethonium-resistant part of the contraction may represent a sympathetic motor response. The augmented contraction response to sympathetic nerve stimulation after small doses of this drug may be ascribed to its known potentiation of responses of effector cells to nerve stimulation (Trigg[?] 1965).

The results obtained after the administration of *propranolol* and *dibenzyline* support the earlier suggestion that the different effects of sympathetic nerve stimulation on the bladder base and the detrusor muscle, i.e. a contraction of the former and an inhibition of the latter, may be ascribed to the action of different effector systems, and not to different nerve fibres. The failure to expel fluid in the presence of a high intravesical pressure evoked by sympathetic nerve stimulation after the administration of propranolol indicates the presence of alpha receptors in the detrusor muscle in addition to the beta-receptors, and at the bladder base as well, since the outlet is closed by the stimulation. The contraction blockade effected by dibenzyline and the abolition of both phases of the response by guanethidine support this theory.

Discussion

The results of the present experiments suggest that the parasympathetic nerves mediate impulses which evoke exclusively motor responses in the bladder. The sympathetic nerve impulses give rise to, on the other hand, an initial contraction of the whole organ supervened by relaxation of the detrusor muscle with persistent contraction of the bladder base. The bladder response to parasympathetic nerve activity is inhibited by sympathetic nerve stimulation. Synergism between the two divisions of the autonomic nerves to the bladder has not been demonstrated by the investigation.

The bladder outlet appears to constitute an internal vesical sphincter as was suggested by Clegg (1957) from anatomical investigations, but the functional significance of this sphincter is not clear since the bladder is still continent after sympathectomy. On the other hand, a sympathetically induced contraction of the bladder base in conjunction with the relaxation of the detrusor muscle gives optimal effects of the sympathetic reflex activity during the collecting phase of the bladder (Edvardsen 1967a).

The mechanisms brought into play by the sympathetic inhibition of the bladder response to parasympathetic nerve activity are not elucidated by these experiments. The sympathetic inhibition of denervated bladders suggests that the action is on the effector cells. This may also be assumed from the sympathetic inhibition of the bladder during its collecting phase, when no background activity is present in the parasympathetic nerves. On the other hand, the sympathetic terminals on parasympathetic ganglion cells in the bladder wall described by Hamberger and Norberg (1965) would suggest sympathetic inhibition of the parasympathetic ganglion transmission as well. It thus seems likely that the sympathetic inhibition is exerted at both the effector and the parasympathetic ganglion level thus allowing maximum efficiency of sympathetic effect both in guarding and continence reflex activity (Garry, Roberts and Todd 1959, Kuru 1965) and in the voluntary interruption of micturition (Edvardsen 1967b).

Referring to the adrenergic receptors, the alpha receptors appear to be located mainly at the base of the bladder but seem also to be distributed in the detrusor muscle. The following observations, made on sympathetic nerve stimulation, are in favour of this latter assumption. (i) The initial bladder contraction can expel more than half the bladder contents when the outlet is kept open by a urethral catheter. (ii) The initial pressure rise, in addition to that already evoked by maximum parasympathetic nerve stimulation, suggests that contraction mechanisms other than cholinergic are involved. (iii) An adrenergic contraction mechanism acting the detrusor muscle is suggested by the guanethidine experiments, where the potentiated motor response following small doses was obtained only on stimulation of the hypogastric nerves, while the response to pelvic nerve stimulation was unaltered. (iv) The raised bladder pressure obtained after the administration of propranolol which is higher than that of spontaneous micturition contraction, indicates distribution of

alpha receptors in the detrusor muscle — In support of this hypothesis is the earlier observation that the bladder depression following intraspinal operative procedures is converted into contraction after the administration of propranolol (Edvardsen 1967c) An initial detrusor contraction following hypogastric nerve stimulation was observed by Langley and Anderson (1895) who employed strips from this muscle, and by Ingersoll *et al.* (Hegre and Ingersoll 1949 Ingersoll, Jones and Hegre 1954, Ingersoll and Jones 1958) who used a cinematographic recording technique with an intact bladder

According to the present experiments the beta receptor is of functional significance in the detrusor muscle only This is deduced from the facts that neither sympathectomy nor the administration of propranolol or guanethidine prevent bladder emptying, and that the bladder base remains contracted during continuous sympathetic nerve stimulation. In support of this hypothesis is the continence of the bladder after blocking the alpha receptors with dibenzyline

From a point of view of function the presence of the alpha-receptors at the bladder base and the beta receptors in the detrusor muscle seems reasonable as regards the storing capacity of the bladder But the significance of the presence of alpha-receptors in the detrusor muscl remains obscure Shabanah, Toth and Maughan (1964) postulated that in the uterus, the beta receptor activity which predominated during pregnancy was changed to alpha-receptor act ity d ring labour and that the time of change-over was determined by the level of progesterone. A change-over from beta to alpha-receptor activity in the urinary bladder has not been disclosed by the present experiments

This work was ponsored by the Norwegian Research Council for Scienc and the Humanities. author is indebted to Professor B R Kaada for generous help and encouragement.

References

Barrington, F J F The nervous mechanism of micturition *Quart J p Physiol.* 1915, 8, 33—71
Burnstock, G G Campbell and J M Rand The inhibitory inner tion of the taenia of the guinea-pig caecum *J Ph iol* Lond 1966 *182* 504- 526
Clegg, E J The musculature of the human prostatic urethra *J Anat* (Lond.) 1957 *91* 345—351
Edvardsen, P Nervous control of urinary bladder in ts I The collecting phase. *Acta physiol scand.* 1968a, 7 157 —1 1
Edvardsen, P Nervous control of urinary bladder in cats II The expulsion phase *Acta physiol sc nd.* 1968b 172—182
Edvardsen, P Nervous control of urinary bladder in ts III Effects of autonomic blocking agents in the intact animal *Act physiol scand* 1968c 7 183—193
Garr R. C., T D Roberts and J K Todd, Reflexes involving the external urethral sphincter in the cat. *J Physiol* (Lond) 1959 149 653—665.
Gjone, R., *A Dual Peripheral and S praspinal Autonomic Influenc on the Urinary Bladder.* Oslo, Universitetsforlaget 1965
Gruber, C. M., The utonomic innervation of the genito-urinary system. *Physiol. Rev* 1933. *13* 497—609.
Hamberger, B. and K. A Norberg, Adrenergic synaptic terminals and nerve cells in bladder ganglia of the cat. *Int. J N pharmacol* 1965 4 41—45

Hegre, E. and E. H. Ingersoll, An analysis of regional variations in the response of the detrusor muscle to electrical stimulation of the hypogastric nerves. *J. Urol.* (Baltimore) 1949 *61* 1037—1047

Henderson, V. E. and M. H. Roepke, The urinary bladder mechanisms. *J. Pharmacol. exp. Ther.* 1935. *54* 408—414.

Hukovic, S., M. J. Rand and S. Vanov, Observations on an isolated, innervated preparation of rat urinary bladder. *Brit. J. Pharmacol.* 1965 *24* 178—188.

Ingersoll, E. H., L. L. Jones and E. S. Hegre, Urinary bladder responses to unilateral stimulation of hypogastric nerves. *J. Urol.* (Baltimore) 1954. *72* 178—190.

Ingersoll, E. H. and L. L. Jones, Effect upon the urinary bladder of unilateral stimulation of hypogastric nerves in the dog. *Anat. Rec.* 1958. *130.* 605—615

Ingersoll, E. H., L. L. Jones and E. S. Hegre, Role of the sympathetic nervous system in urinary bladder function in the cat. *Neurology* 1961 *11* 46—55

Jansson, G. and J. Martinson, Some quantitative considerations on vagally induced relaxation of the gastric smooth muscle in the cat. *Acta physiol. scand.* 1965. *63* 351—357

Koikegami, H., T. Dodo, Y. Mochida and H. Takahashi, Stimulation experiments on the amygdaloid nuclear complex and related structures. Effects upon the renal volume, urinary secretion, movements of the urinary bladder, blood pressure and respiratory movements. *Folia psychiat. neurol. jap.* 1957 *11* 157—206.

Kuru, M., Nervous control of micturition. *Physiol. Rev.* 1965. *45* 425—494

Kuru, M. and T. Iwanaga, Ponto-sacral connections in the medial reticulo-spinal tract subserving storage of urine. *J. comp. Neurol.* 1966. *127* 241—265

Kuru, M., H. Ozaki and F. Kurati, Effect of simultaneous stimulation of the bulbar vesicoconstrictor and vesico-relaxer centers. *J. comp. Neurol.* 1961 *116* 195—208.

Langley, J. N., The effects of various poisons upon the response to nervous stimuli chiefly in relation to the bladder. *J. Physiol.* (Lond.) 1911 *43* 125—181

Langley, J. N. and H. K. Anderson, The innervation of the pelvic and adjoining viscera. *J. Physiol.* (Lond.) 1895 *19* 71—139.

Plum, F. and R. H. Colfelt, The genesis of vesical rhythmicity. *Arch. Neurol.* (Chic.) 1960 2. 487—496

Root, M. A., Effect of tetraethylammonium chloride on the urinary bladder of the cat. *Fed. Proc.* 1947 *6* 366.

Shabanah, E. H., A. Toth and G. B. Maughan, The role of the autonomic nervous system in uterine contractility and blood flow. *Amer. J. Obstet. Gynec.* 1964 *89* 841—880.

Sherrington, C. S., Postural activity of muscle and nerve. *Brain* 1915 *38* 191—234

Saso, E. B. and T. D. Saso, Sympathetic stimulation and blockade of the urinary bladder in cat. *Int. J. Neuropharmacol.* 1964. *3* 241—251

Triggle, D. J., *Chemical Aspects of the Autonomic Nervous System.* London and New York, Academic Press 1965 pp. 329

Ursillo, R. C. and B. B. Clark, The action of atropine on the urinary bladder of the dog and on the isolated nerve-bladder strip preparation of the rabbit. *J. Pharmacol. exp. Ther.* 1956. *118* 338—347

Wang, S. C. and F. Harrison, The nature of bladder responses following stimulation of the anterior hypothalamus. *Amer. J. Physiol.* 1939 *125* 301—309

Acta physiol. scand 1968 72. 248—252

F om the Department of Pharmacology Un ity of Lund, S eden

Effects of Phospholipase A and Lysolecithin on Some Electrical Properties of the Muscle Membrane

B

E. X. Albuquerque[1] and S Thesleff

Rec ed 25 June 1967

Abstract

Albuquerque, E. X. and S Thesleff *Eff t f pho ph lipase A and lysolecithin on e lectrical pr pe tie f th m le m mb an* Acta ph siol. scand. 1968. 7 48—25

The ffects of incubation w th phospholipase A and l solec thin on the resting membrane potential action potential inpu esistanc and acetylcholine sensitivi of isolated extensor digi orum longus and soleu muscles of he wer compared with those previousl obtained with phospholipase C A common ac ion of the nze s studied w re tha they in sufficient doses depolarized the mu le fibres and reduced the inpu esistanc I contrast to phospholipase C which irreversibl blocked action potential generation, phospholipase A and solec thin had lit le effec on membr ne ex itabili The sensitiv t of hronicall denervated scle fibres to locall pplied acetylcholine was unaffected by the tw phospholipases and by lecithin The differenc in action between phospholipase C and phospholipase A on electrogenic membrane excitabili is suggested be explained by the respectiv mode of lipolytic action of the o enzymes Phospholipase C hydrol ses the d gl eride — phosphate ester linkage in phosphatides nd he eby emoves the phosphate head from the molecule hile phospholipase A leaves one fat acid ester linkage leaving the polar heads of phospholipids intact. L solec thin possibl rearranges meml ane struc ures by ts high su face activity

The irreversible blockade of th act potential generating mechanism, by phospholipase C (PhC and the sim ltaneou h nges in the resting potential and the ionic permeabilit of the skeletal muscl membrane ha recentl been described by Albuquerque and Thesleff (1967a, b It wa suggested that PhC affected the membrane by enzymic h drol ses of the ester linkage between the α-carbon and phosphate groups of some pecific membraneous phospholipids which are considered to be invol ed in passi e ionic transport (Mullins 1961 Goldman 1965, Blaustein and Goldman 1966) However the possibil t remained that the effects of PhC were secondar to a more unspecific rearrangement of membrane structures. For

[1] Research F llow of IBRO/Unesco Presen address Department of Pharmacology State Uni ersity of New York t Buffalo, Buffalo, New York 14214 USA.

that reason we decided to explore the effects on the muscle of another lipase which also hydrolyses membrane phosphatides but which contrary to PhC leaves the phosphate groups intact. The enzyme used was phospholipase A (PhA) which hydrolyses one fatty acid ester linkage in phosphatides to form lysophosphatides. Since lysophosphatides in themselves are powerful membrane-destroying agents, lysolecithin was also included in the study. As in our previous investigation with PhC the present experiments deal with the effects on the resting membrane potential, input resistance, acetylcholine sensitivity and on some of the properties of the action potential in isolated extensor digitorum longus and soleus muscles of the rat.

Methods

The experiments were made in vitro using the innervated or chronically denervated extensor digitorum longus and the soleus muscles of young adult rats. The experimental conditions and the electrical recording techniques have been described by Albuquerque and Thesleff (1967a, b).

The Krebs-Ringer solution had the following composition in mM: NaCl-135.0, KCl-5.0, $NaHCO_3$-15.0, NaH_2PO_4-1.0, $CaCl_2$-2.0, $MgCl_2$-1.0 and Glucose 11.0. The temperature of the solution was maintained at 25° C and it was continuously oxygenated with a gas mixture of 95 % O_2 and 5 % CO_2 which gave it a pH of 7.0—7.3.

Immunoelectrophoretically pure phospholipase A (phosphatide acylhydrolase, EC 3.1.1.4) from bee venom was generously supplied by Dr B. Hogberg, Leo, Halsingborg, Sweden. Lysolecithin was obtained from Koch & Light Laboratories, England. The doses of the enzyme and of lysolecithin added to the bathing solution are expressed in μg/ml of the total material.

In general the experiments were made on two muscles, the extensor and the soleus, mounted together in the bath. The time of incubation with the enzyme or with lysolecithin was 60 min and after that period the recordings were made without a change of bathing solution. The time required for the measurements was 10—20 min making the total time of incubation no longer than 80 min.

Results

As shown in Table I incubation for 1 hr with PhA or lysolecithin in logarithmically spaced doses from 6.25 to 50 μg/ml progressively reduced the resting membrane potential of muscle fibres. Simultaneously the ionic permeability of the muscle membrane increased as shown by the fall in the input resistance of muscle fibres. The extensor and the soleus muscle were about equally sensitive to these effects of PhA and lysolecithin, but in the case of PhA considerable differences existed between individual muscle fibres as shown by the large standard deviation of the means observed with the high doses. For comparison, the values obtained in the study by Albuquerque and Thesleff 1967a with 1.5 μg/ml of PhC are also shown in the table.

When the effects of PhA and lysolecithin on action potential generation were examined we used doses which did not depolarize the fibres below a resting membrane potential of 50 mV. To diminish differences in excitability caused by variations in the resting membrane potential the fibres were polarized to about 90 mV by the passage of a constant anodal current of 100 msec duration. Besides the recording of the amplitude of the action potential, the threshold potential at which the spike was generated was also measured. The results obtained are shown in Table II

TABLE I Effects produced by PhA, lysolecithin and PhC on the resting membrane potential and input resistance of innervated extensor digitorum longus (extensor) and soleus muscles. The resting membrane potential and the input resistance are expressed as mean ± S.D. The figures in parentheses show the number of fibres analysed

	Extensor		Soleus	
	Resting Membrane Potential mV	Input Resistance 10^6 Ω	Resting Membrane Potential mV	Input Resistance 10^6 Ω
Control	74 5.5 (75)	7 ± 3.3	69 ± 3.7 (75)	6 ± 2.4
Dose μg ml	PhA			
6.25	53 ± 10 1 (6)	7 3.1	66 ± 3.3 (6)	7 ± 0.5
12 5	52 ± 7.3 (7)	6 1.0	58 ± 4.0 (3)	6 ± 1 4
25 0	17 ± 16 7 (6)	3 ± 7.3	—	—
50.0	10 ± 16.2 (3	3 6 7	7 ± 5.1 (6)	4 ± 3.1
	Lysolecithin			
6.25	61 6.8 1	5 1 0	65 ± 3.8 (12)	6 ± 1.3
12.5	50 5.9 (10	4 ± 1 6	62 ± 6.7 (11)	5 ± 0.9
5.0	3 8 5 6	4 2 1	48 ± 13.0 (6)	7 ± 1.6
50.0	34 20.0 3	3 1 4	9 ± 8.6 (3)	3 ± 1.3
	PhC			
1 5	57 5 7 11	5 1.2	67 ± 3.6 (12)	5 ± 0.9

TABLE II The action of PhA, lysolecithin and PhC on the action potential generation and resting membrane potential of the innervated extensor digitorum longus (I) and soleus muscle (II). The resting membrane potential and action potential values are the mean ± S.D. The figures within parentheses depict the number of fibres studied

		Resting Membrane Potential mV	Action Potential Threshold mV	Action Potential Amplitude mV
Control	I	75 4 0 50	6 ± 2 2	96 ± 9.3
	II	71 5 0 44	26 ± 3.2	82 ± 17.0
Dose μg ml		PhA		
	I	61 4 5 12	36 ± 5.0	70 ± 19.0
12.5	II	63 5 4 12	29 ± 3 5	72 ± 13.8
		Lysolecithin		
	I	59 ± 5 4 (12	36 ± 15 5	67 ± 17 1
6.25	II	67 ± 5 5 (12	33 ± 8.4	71 ± 1.7
	I	55 6 9 (7	41 ± 8 8	67 ± 19.2
12.5	II	55 ± 6.0 (10	45 ± 7 0	56 ± 17.0
		PhC		
	I	55 ± 11.2 (107)	5 87	
1.5	II	63 ± 10 (25)	0/25	—

Action potentials were elicited in 5 fibres out of 87

TABLE III. Effects of PhA and lysolecithin on the ACh-sensitivity of the chronically denervated extensor digitorum longus (I) and soleus muscles (II). The resting membran potential is the mean ± S.D. in mV. The figures within parenthesis are the number of fibres studied. ACh-sensitivity is expressed as the mean ± S.D. of the ratio of membrane depolarization potential produced by ACh to the charge flowing through the ACh pipette (for details see Albuquerque and Thesleff 1967)

		Resting Membrane Potential mV	ACh-sensitivity
Control	I	55 ± 3.1 (35)	5 ± 2.3
	II	61 ± 3.0 (35)	6 ± 3.2
Dose μg/ml		PhA	
	I	39 ± 7 1 (15)	2 ± 0.8
	II	53 ± 6.1 (11)	3 ± 2.3
	I	35 ± 7.9 (8)	1 ± 0 9
	II	43 ± 9.0 (12)	2 ± 1.8
		Lysolecithin	
	I	50 ± 5.4 (12)	3 ± 1.6
12.5	II	52 ± 4.6 (12)	5 ± 1 7

which, for comparison, includes the effects of PhC as observed in the extensor digitorum longus by Albuquerque and Thesleff (1968b). It is evident that neither PhA nor lysolecithin in the doses used were abl to abolish membrane excitability even if they reduced the amplitude of the self regenerative response and increased the threshold for spike generation. This finding was in marked contrast to PhC which in a dose of 1 5 μg/ml completely abolished the action potential.

The effects of the enzymes and of lysolecithin on the acetylcholine sensiti ity of the membrane were examined in chronically denervated muscles by iontophoretic microapplication of acetylcholine to the cell surface and the recording of the resulting membrane depolarization. As shown by the mean values for acetylcholine sensitivity presented in Table III the responsiveness of the membrane to applied acetylcholine was relatively little affected by the agents. One may conclude that PhA and lysolecithin, similarly to PhC (Albuquerque and Thesleff 1967a) are not directly interfering with the receptor or the acetylcholine-receptor interaction.

Discussion

The present study revealed that PhA, PhC and lysolecithin produced in the mammalian muscle membrane the same kind of changes as those previously described in the membrane of frog muscle, squid and lobster giant axons (Tobias 1955 Narahashi 1964) i.e. depolarization and an increase in the resting ionic permeability. The effects of PhA were presumably due to the action of the enzyme on lipoprotein-

bound phospholipids causing a cleavage of one fatty acid ester linkage (Deenen 1965) while with PhC the phosphate head was removed from the molecule. Since phosphate groups of phospholipids are believed to be involved in passive ionic transport (Tobias, Agin and Pawlowski 1962, Goldman 1964, Blaustein and Goldman 1966) it is reasonable to assume that the primary effect of PhC is to block the action potential while with PhA this effect is a secondary one. Thus, PhA would depolarize the membrane without a direct action on spike generation.

Lysolecithin, a product of the hydrolytic action of PhA on lecithin, produced effects similar to PhA. Although the mechanism of action of lysolecithin upon the membrane is unknown, it is possible that it due to its high surface activity could dislodge membrane lipids and thereby rearrange the membrane structure (Habermann 1955). The results obtained support the suggestions that the action of PhC on the action potential generating mechanism is a direct consequence of its interference with the integrity of hydrophilic heads of certain membrane phospholipids (Thesleff and Albuquerque 1967, Albuquerque and Thesleff 1967a, b).

The study was supported in part by research grants from the Swedish Medical Research Council (14 738-01) Stockholm, Sweden, and the Royal Physiograph Society of Lund, Lund, Sweden. We are greatly indebted to Dr. B. Hogberg of AB Leo, Halsingborg, Sweden for generous supply of phospholipase A.

References

Albuquerque, E. X. and S. Thesleff: Influence of phospholipase C on some electrical properties of the skeletal muscle membrane. J. Physiol. (Lond.) 1967a. 190: 123–137.
Albuquerque, E. X. and S. Thesleff: The effect of calcium on the skeletal muscle membrane after treatment with phospholipase C. Acta physiol. scand. 1968b. 72. In press.
Blaustein, M. P. and D. E. Goldman: Competitive action of calcium and procaine on lobster axon. A study of the mechanism of action of certain local anesthetics. J. gen. Physiol. 1966. 49: 178–191.
Deenen, L. L. M.: Phospholipids and biomembranes. Progress in the chemistry of fats and other Lipids. Ed. Holman, R. T. N. Y. Pergamon Press. 1965. 8: 1–127.
Goldman, D. E.: A molecular structural basis for the excitation properties of axons. Biophys. J. 1964. 4: 167–188.
Goldman, D. E.: Gating control of ion fluxes in axons. J. gen. Physiol. 1965. 48: 75–77.
Habermann, E.: Über Permeabilitätsänderungen durch tierische Gifte. Arch. exp. Pathol. Pharmacol. 1955. 225: 158–160.
Mullins, L. J.: The macromolecular properties of excitable membranes. Ann. N. Y. Acad. Sci. 1961. 94: 390–404.
Narahashi, T.: Restoration of action potential by anodal polarization in lobster giant axons. J. cell. comp. Physiol. 1964. 64: 73–96.
Thesleff, S. and E. X. Albuquerque: The cholinergic receptor systems of denervated striated muscle. Ann. N. Y. Acad. Sci. 1967. In press.
Tobias, J. M.: Effects of phospholipase, collagenase and chymotrypsin on impulse conduction and resting potential in the lobster axon with parallel experiments on frog muscle. J. cell. comp. Physiol. 1955. 46: 183–207.
Tobias, J. M., D. P. Agin and R. Pawlowski: Phospholipid-cholesterol membrane model—control of resistance by ions or current flow. J. gen. Physiol. 1962. 45: 989–1001.

Acta physiol. scand. 1968. 72. 253—254

From the Institute of Anatomy and Histology, University of Lund, Sweden

A Possibility for Differentiating Dopamine from Noradrenaline in Tissue Sections by Microspectrofluorometry

by

Anders Björklund, Berndt Ehinger and Bengt Falck

In the histochemical fluorescence method of Falck and Hillarp (see Falck and Owman 1965) the fluorophores formed from dopamine (DA) and noradrenaline (NA) have very similar fluorescence characteristics, and thus cannot be distinguished from each other in the microscope (Falck 1962). The work preliminarily reported here aims at obtaining methods for their distinction in the microscope or microspectrograph.

Parallel studies were performed on protein microdroplets (1 mg/ml DA-hydrochloride and NA-bitartrate dissolved in water or 0.15 M phosphate buffer pH 7.5 1—4 % human serum albumin was added) and tissue sections. The DA fluorophore was studied in varicose nerve fibres in the pedal ganglion of the fresh water bivalve, *Anodonta piscinalis* and bovine liver mast cells. The NA fluorophore was studied in adrenergic nerves of the bovine liver vessels and guinea pig vas deferens. Changes in pH were induced by exposing the models and the non-deparaffinized sections to HCl-vapor at room temperature. The sections were mounted in liquid paraffin.

After only formaldehyde treatment both DA and NA show a broad excitation peak at about 410 mμ. After short exposure to HCl vapor (about 15 sec) both DA and NA show shift of this peak to about 370 mμ (*cf.* Caspersson, Hillarp and Ritzén 1966). A similar shift has been observed in the UV absorption maxima of the fluorophores upon acidification (Corrodi and Hillarp 1964). Upon longer or even severe HCl treatment the excitation spectrum of DA does not change any further. In the case of NA, however, the above-mentioned peak at the shorter wavelength will quickly diminish and an excitation peak at approximately 330 mμ will increase (*cf.* Corrodi and Jonsson 1965). The observed differences strongly suggest that treatment with HCl can be used to differentiate DA from NA in tissue sections. However, marked differences in behavior between differently located amines have been observed. The section thickness may also influence the speed and completeness of the reaction. These observations necessitate proper standardization of the procedure for various tissues.

This research was supported by grants from the Swedish Medical Research Council (B68-12X-1-03B) and from the United States Public Health Service (NB-06701-01).

References

CASPERSSON, T., N. Å. HILLARP and M. RITZÉN: Fluorescence microspectrophotometry of cellular catecholamines and 5-hydroxytryptamine. *Exp. Cell Res.* 1966, 42, 415—428.
CORRODI, H. and N. Å. HILLARP: Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 2. Identifizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd. *Helv. chim. Acta* 1964, 47, 911—918.
CORRODI, H. and G. JONSSON: Fluorescence methods for the histochemical demonstration of monoamines. 4. Histochemical differentiation between dopamine and noradrenaline in models. *J. Histochem. Cytochem.* 1965, 13, 484—487.
FALCK, B.: Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. *Acta physiol. scand.* 1962, 56, Suppl. 197.
FALCK, B. and CH. OWMAN: A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta Univ. Lund.* Sectio II 1965, No. 7, 1—23.

Acta physiol. scand. 1968. 72. 255—256

From the Department of Physiology, Karolinska Institutet, Stockholm, Sweden

Occurrence and Some Properties of Dopamine Containing Granules in the Sheep Adrenal

By

F. Lishajko

In 1951 Goodall discovered that in addition to adrenaline (A) and noradrenaline (NA) small amounts of dopamine (DA) were present in the sheep adrenal. Shortly afterwards Shepherd and West (1953) found DA also in bovine adrenals. After Blaschko and Welch (1953) and Hillarp *et al.* (1953) had localized A and NA in cytoplasmic granules of the chromaffin cells, Eade (1958) was able to show that the DA present in bovine adrenals was bound to a "large-granule" fraction. The present report deals with the occurrence of DA in sheep adrenal medullary granules and describes some of their properties.

Adrenal glands were obtained from the slaughter house and transported at 0° to the laboratory within 30—60 min after the death of the animal. All the subsequent preparations were made at 0—5°. The medulla was homogenized in isotonic potassium phosphate at pH 7.5. The suspension was centrifuged at 1000 × *g* for 10 min and the supernatant recentrifuged at 50,000 × *g* for 20 min. The high speed sediment was washed with phosphate buffer and the pellet extracted with 0.4 N-perchloric acid. After adjustment of the extract to pH 4 with N—KOH and removal of the precipitate, the extract was used for chromatographic separation on Amberlite CG 120 type II (Häggendal 1962) in Na form and eluted with N—HCl. An aliquot of each fraction was read in an Aminco spectrophotofluorimeter at 285 mμ (activation) and 335 mμ (fluorescence) (Fig. 1).

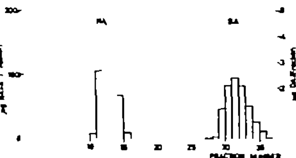


Fig. 1. Fractions of high speed sediment extract from sheep medullary granules obtained from an Amberlite cation exchange resin. The chromatogram represents NA + A in fractions 12—15, and DA in fractions 29—35. Readings made at 285 mμ (act.) and 335 mμ (fluoresc.).

TABLE 1 Isolated adrenal medullary granules incubated 60 min in K-phosphate at pH 7.5 and 37 Addition as indicated. Amine concentrations in medium of the control at end of incubation per ml DA, 0.2–0.8 10 M NA, 2–3 10 M A, 4–7 x 10 M

	Per cent remaining in sed. after incubation		
	DA	NA	A
Control	22	46	44
DA, 3 10 M	34	56	56
ATP Mg 3 mM	46	56	71
ATP Mg, 3 mM DA 3 10 M	91	4	64

Number of stars denote significance of difference from control

Th esult confirm and extend the previous esults of Eade (1958) concerning the occurrence of DA in medullary granules

The washed nd resuspended sediment from 30,000 g on incubation for 60 min at 37 in iso tonic potassium phosphate at pH 7.5 showed a higher release rate for DA than f r NA and A indicating either binding to different storage particles or to different torage ites

Addition of DA 3 10 M or ATP Mg[2] 3 mM or both, decreased the net re lease f DA uggest ng ncreased reuptake (Table I) Similarly addition of TP—Mg and DA 3 10 M to partially depleted granules (not shown in the greatly enhanced th net DA uptake This ffect is blocked by reserpine M On th other hand no net uptake of NA and A was observed in partially depleted gran les during incubation in the presence of ATP—Mg and the amines in 2—5 10 ncentration.

The resear h por ed his doc ment has been sponsored in part by the *AIR FORCE OFFICE OF SCIENTIFIC RESEARCH* through the European Offic of Aerospace Research, OAR, United States A Forc nder G ant AF EOAR 67 18.

References

BLASCHKO H and A D WE Localization of adrenaline in cytoplasmic particles of the bovine adrenal medulla *A h p Path Ph mak* 1953 *219* 17—22

EADE N R The distribution of the atechol amines in homogenates of the bovine adrenal medulla. *J Physiol Lo d* 1958 *141* 183 192

GOODALL McC Studies of adrenaline and noradrenaline in mammalian heart and suprarenals. *Act physiol scand* 1951 *24* Suppl 85

HAGGENDAL J On the use of strong exchange resins for determinations of small amounts of catechol amines. *Sca d J cli Lab I est* 1962 *14* 537 544

HILLARP N A S LAGERSTEDT and B NILSON The isolation of a granular fraction from the suprarenal medulla, containing the sympathomimetic catechol amines. *Acta physiol scand* 1953 *29* 251—263

SHEPHERD D M and G B WEST Hydroxytyramine and the adrenal medulla. *J Physiol (Lond.)* 1953. *120* 15—19

Acta physiol. scand. 1968. 72. 257—281

From the Department of Physiology and the Department of Histology University of Goteborg Sweden

On the Components of the Kr^{85} Wash-Out Curves from the Small Intestine of the Cat

By

Mogens Kampp Ove Lundgren and Johan Sjöstrand

Received 23 June 1967

Abstract

Kampp M., O Lundgren and J Sjöstrand *On the components of the Kr^{85} wash out curves from the small intestine of the cat* Acta physiol. scand. 1968. 72 257—281

The wash-out of intra-arterially injected Kr^{85} from denervated small intestinal segments (mainly jejunum) of the cat was recorded by means of scintillation detector at various levels of vascular tone The elimination curve could be resolved into four components (I IV) at all blood flow levels. Four independent methods were used in an attempt to localize the different components, namely registration of the elimination of β activity local injections into different parts of the intestinal segment, autoradiography and comparisons of weights of the compartments. The following localization of the four components was made probable. The first component reflected predominantly countercurrent exchange of krypton between the ascending and descending limbs of the vascular loops in the mucosa at resting flow levels. During vasodilatation, this component mirrored mainly the wash-out of the tracer from well-perfused region in the mucosa-submucosa. The second and third components mainly reflected the disappearance of Kr^{85} from the mucosa and the muscularis, respectively The slowest component (IV) probably mirrored an elimination of Kr^{85} from the perivascular fat of the mesentery and an absorption of the tracer from the intestinal lumen

Most investigators studying the blood flow of the small intestine have been concerned with total blood flow of the tissue (for reviews, see Grim 1963 Grayson and Mendel 1965) In a few investigations, attempts have been made to ascertain the distribution of blood within the intestinal wall by injecting intra vascularly microspheres labelled with Na (Grim and Lindseth 1958) Rb^{86} (Csernay Wolf and Varro 1965) or DHO (Rayner McLean and Grim 1960 Wenner 1961 Weiner and Grim 1966) with a subsequent analysis of the amount of tracer present in the different layers of the intestinal wall. Apart from the obvious disadvantage that only one experimental run can be performed on each animal, there are certain theoretical objections to these methods.

When microspheres are injected, the blood flow fraction thought to pass through non-nutritional shunt vessels is probably overestimated, since some microspheres (diameter 12 μm) will in all probability pass such capillary exchange vessels where

the diameter exceeds 1? µm (*cf* Folkow 1967) On the other hand, when the two above-mentioned lipid insoluble tracers are used, the nutritional blood supply to tissues with comparatively low blood flow rates is probably overestimated since the extraction of DHO and particularly of Rb^{86} becomes more complete the slower the linear rate of flow (*cf* Renkin 1959 Lassen 1964 Dresel, Folkow and Wallentin 1966, Pitts 1967) Furthermore it has been demonstrated that the rate of wash-out of lipid insoluble substances from cat skeletal muscle is considerably influenced by factors other than blood flow e.g. by the rate of net transcapillary fluid movement (Lundgren and Mellander 1967)

The use of easily diffusible lipid soluble inert substances such as hydrogen, krypton or xenon for studies of blood flow distribution seems on theoretical grounds more satisfactory (*cf* Landis and Pappenheimer 1963) Such tracers also offer the advantage that blood flow distribution may be repeatedly determined in one and the same experimental animal. Methods utilizing the aforementioned inert gases have recently been developed for quantitative measurements of blood flow distribution in several tissues, such as the brain (Ingvar and Lassen 1962, Häggendal Nilsson and Norbäck 1965) the kidney (Thorburn *et al* 1963 Aukland, Bower and Berliner 1964 Ladefoged *et al* 1965) and the myocardium (Herd *et al* 1962, Aukland, Bower and Berliner 1964 Ross *et al* 1964)

In the present series of experiments attempt have been made to study the blood flow distribution in the small intestine of the cat by analysing the nature of the composite wash-out curve which can be recorded by an external scintillation detector after close arterial slug injections of $krypton^{85}$ The present paper presents the results obtained with the different techniques used for localizing the four com- nts of the externally registered disappearance of activity In a subsequent the blood flow distribution within the intestinal wall will be analysed in relation the total venous outflow from the intestine Preliminary reports of these studies have been published previously (Kampp and Lundgren 1966a, Lundgren and Kampp 1966) and the general results hav been summarized by Folkow (1967)

Methods

A Gen ral principle

The theoretical considerations below are largely based on Kety's pproach to tissue-blood exchange of inert gases and the reader is eferred to his reviews for more detailed discussions (Kety 1951 1960)

The limination of an inert gas from constantly and uniformly perfused tissue can be described by the following equation provided that the arterial concentration of the gas is zero or negligibly low during the wash-out period

$$C_t = C_0 \quad ^{kt} \tag{1}$$

where C_t and C_0 denote the tissue concentration of the gas at times t nd 0, and k denotes the learance constant. The constant, k, which is closely related to the blood flow (see below) can be determined from the mathematically derived equation

$$k = \frac{1 \quad 2}{t\ 1/2} \tag{2}$$

where t 1/2 denotes the half time of decay in min t 1/2 is calculated from the straight line obtained when plotting equation (1) on semilogarithmic paper

If the disappearance of gas from two or more parallel-coupled, homogeneously perfused tissues with different rates of desaturation is simultaneously registered, the resulting elimination curve is the sum of the single exponentials according to the general formula

$$A_t = A_{1_0} \quad ^{k_1t} + A_{2_0} \; e^{-k_2t} + A_{3_0} \quad ^{k_3t} + \quad + A_{n_0} \quad ^{k_nt} \qquad (3)$$

(A_t = total counts per min at time t A_{1_0}, A_{2_0}, A_{3_0} A_{n_0} = the number of counts per min initially present in each component k_1, k_2, k_3 k_n = clearance constants of the different components) If A_t is plotted semilogarithmically *versus* time an arched curve is obtained. Provided n is low and the k-values differ enough, it is possible to resolv this composite curv into its different components by means of the method orginally described by Dobson and Warner (1957) A straight line is then drawn through the terminal straight (uniexponential) portion of the curv and extrapolated to time zero. This straight line is then subtracted from the original curv and line is again drawn through the terminal traight part of the constructed curve. The procedure is repeated until a final straight line is obtained. When the number of components (n in equation 3) is large, the disappearance curv may still be resolved into 2—5 components, which under some circumstances may represent physiologically meaning ful averages. Each component may for example, reflect an average also of range of blood flows in separat tissue compartments within which flow is not entirely uniform (*cf* Kety 1963 Häggendal, Nilsson and Norbäck 1965 Linder 1966) Upon analysis of the wash-out curv in the manner described above, correct estimations of the respective *k*-values of the different components can be obtained only if it can be assumed that negligible diffusion of the tracer takes place between the different compartments.

If the gas leaves tissue compartment exclusively via the blood, and if it can be assumed that gas equlibrium between blood and tissue is reached in fraction of a second, i negligible concentration gradients exist in the tissue, then blood flow (f) of the tissue can be calculated in ml/min × 100 g from the formula

$$f = k \quad 100 \qquad (4)$$

Here denotes the tissue blood partition coefficient of the gas divided by the specific weight of the tissue. Experimental data, validating the assumption of blood flow-limited wash-out of inert gases, hav so far been presented for the myocardium (Aukland, Bower and Berliner 1964 Ross *et al.* 1964) the white matter of the brain (Kety 1965) the renal cortex (Ladefoged *et al.* 1965) and fat tissue (Larsen, Lassen and Quaade 1966) In addition, the data of Jones (1950) may indicate general pplicability of the assumption.

In the case of simultaneous registration of several elimination rates (multiexponential curves) the relativ fractions of the total flow that corresponds to each individual curve component is estimated in the following way *provided that the activity of all these curve components is recorded with equal efficiency* The amount of tracer present in each component at the end of slug injection (A_0 in equation 3) is determined by extrapolating each component to time zero. By relating A_0 to the total amount of the tracer the relative amount of blood flow distributed to each compartment can be calculated.

B. *Operativ proc dur*

The experiments were performed on 44 cats, anaesthetized intravenously with chloralose (40—70 mg/kg) *after induction with ether The cats had been deprived of food for at least* 24 hours and had no obvious signs of intestinal infection

After insertion of tracheal cannula, the abdomen was opened in the midline and the greated omentum and the spleen were extirpated. A segment of the small intestine, weighing 20—50 g (mainly jejunum) was chosen for the experiment and the remainder of the intestinal trac was extirpated Without interfering with the ascular supply to the intestinal segment par of the lymph nodes around its mesenteric root was excluded from the blood circulation by ligatures. The lumen of the jejunal segment was flushed with body-warm Tyrode solution or physiological saline is polyethylene tubes inserted in both ends of the segment, until the fluid leaving the intestinal lumen was clear During this procedure great care was taken to avoid any air entering the segment Most of the fluid remaining in the lumen after this rinsing, was gently squeezed out. The influence of the sympathico-adrenal system was eliminated by cutting the splanchnic nerves bilaterally and by denerv ting the lef adrenal gland and excluding the right one from blood circulation by ligatures The agal influence was liminated by giving atropine 1 1 mg/kg

After heparinizing the cat (3—5 mg/kg) the right femoral artery was cannulated and connected to mercury manometer to record mean arterial blood pressure. The superior mesenteric ein, draining all the blood from the isolated intestinal segment and its remaining lymph nodes, was cannulated and connected to an optical drop-recorder unit (Lindgren 1958)

operating an ordinate write recording on smoked kymograph pape The venous outflow pressure from the intestine ould be set at any desired lev l by adjustments of the end of the tube draining from the drop-recorder This pressure was usually kept at level of approximately 5 mm Hg. Venou blood emerging from the end of this tube was collected in funnel nd returned to the animal ia catheter in the right jugular ein A small branch of one of the mesenteric rteries supplying the intestine was annulated with thin polyethylene thete (PE 10) to permit lose intra-arterial injections of the tracer

In order to secur constant geometrical onditions during the radioactiv measurements, the intestinal segment was placed on specially designed wooden plate outside the abdominal cavity The temperature of the intestine was continuously controlled with a thermocouple thermometer (Elektrolab, Copenhagen, type TE 3) in the lumen of the gut and was kept at 38 C, by means of heating lamps. The serosal surfac of the intestine and the major part of its mesent ry was covered by Mylar® (12 μm thick Du Pont) to hinder diffusion of the radioactiv gas from tissue to air Parts not overed by Mylar were protected by gauze soaked with body-warm saline.

The wash-out of Kr^{85} from the intestine was registered t various levels of intestinal blood flow as continuously recorded by the drop-recorder unit. T induce graded levels of intestinal asodilatation constant infusions of isopropylnoradrenaline were made either intravenously a cathete in the left femoral ein or lose intra-arterially through catheter inserted in retrograde fashion int the superior mesenteric artery This asodilator drug was utilized to relaxe the intestinal smooth muscles as well as the scular smooth muscle (f Fellow Lundgren and Wallentin 1963) It was given in solutions ontaining 10 or 50 μg/ml, at constant rate of 0.1—0.3 ml/min.

Bleeding from animals was refully oided during the operative procedures. If blood losses ocurred, they w substi ted by intra enous of sions of body-warm Dextran solution. Such transfusions of the plasma substit t were sually made between experimental runs in order to avoid sudden hanges in hematocrit which, in t rn, would affect the tissue-blood partition coefficient of Kr^{85} (cf Kety 1951) The temperature of the cat was maintained constant at 38 C by means of hermostatically regulated he ting pad.

At the end of each experiment the weight of the intestinal preparation was determined to allow for alculations of blood flow in ml/min × 100 g I most experiments the muscularis was dissected free from the mucosa-submucosa and weighed separately The weight of the mesentery was also determined in some experiments

C Int -arterial jections of K 85

4—0.9 ml of 0.9 per cent saline solution containing Kr^{85} (about 0.8 mC/ml purchased from the Radiochemic l Cente Amersham, England) was given through the thin polyethylene itheter (see section B) as single injection, lasting 1 — seconds Control injections of Ev blue demonst ated that no significant amounts of the injected dy reached the lymph nodes round the mesen eric root, when administered in this way The temperature of the injected solution was held t 38 C to oid intestinal asoconstriction to cooling. After the slug injection of the tracer the theter was allowed to fill with arterial blood. In control experiments it was shown that the decrease of γ-activity registered during this procedure was insignificant (about 1 per cent of the initial activity)

In most experiments the wash-out of Kr^{85} from the intestine was registered by three detectors simult neously The γ-emission of Kr^{85} (0.6 per cent of the total radioactivity of Kr^{85}) was recorded by scintillation detector with a thallium-activ ted NaI-crystal (size 1 3/4 × 2") placed 4—6 cm from the intestine w th the front surface of the crystal parallel to the wooden support of th intestine I order to ensure that radioactivity from only the intestine and its mesentery was registered by the scintillation detector the whole abdomen except the intestine was covered by lead plat 5 mm thick Further lead plate 5 or 10 mm thick was placed between the thorax and the detector Contamination of the air in the room with Kr^{85} was reduced by continuously suctioning int hood the animal' expired air and the air around the funnel in the jugular ein

The scintillation detector was coupled t spectrometer (Packard A to-Gamma Series 410 A) and linea ra emeter (Packard Model 386) operating one channel of three channel Ink-writer (Rika Denki, Kog Model 34 B) Pulses corresponding to γ-engeries abo 70 Kev were counted In the initial phase of an experimental run, when the change of radioactivity was rapid, the time constant of the ratemeter was set at 0.3 sec and the paper speed of the recorder t 20 cm/min These parameters were then changed in stepwise fashion to 30 sec and 1 cm/min when the tail of the wash-out curve was reached.

The β-activity of Kr^{85} was recorded externally by Geiger Müller end window tube (Philips No. 18504 diamete of window 9 mm) completely enclosed in lead (5 mm thick) except for the window The tube was placed 2—5 mm from the serosal surface of the intestine at the

antimesenteric border Since 90 per cent of uniformly distributed Kr^{85} activity recorded by the external G-M tube, emanates from the outer 0.7 mm of the intestine wall (Thorburn, Casey and Molyneux 1966) this probe mainly registered radioactivity from the intestinal serosa and the muscularis. The G-M tube was coupled to a linear ratemeter (Packard Series 280 A) operating second channel of the Rika Denki recorder used for the registration of γ-activity In manner similar to that described above the time constant of the ratemeter was hanged stepwise from 1 sec to 30 sec. When counts exceeded 5 000/min correction was made for coincidence loss, using alue of 100 microsec fo the dead time of the G-M tube. The oltage characteristics of the tube was regularly checked.

The β-emission of Kr^{85} was also registered in the intestinal lumen by cylindrical G-M tube (Philips No. 18509 length about 20 mm, largest diameter 7 mm) inserted into that end of the intestine, which was closest to the catheter used for injection of Kr^{85} Most of the radioactivity registered by this probe emanated from Kr^{85} in the illi and the intestinal lumen (see abov) The tube was coupled with "digital" ratemeter which measured counting rates in fixed time intervals, and the ratemeter was connected t the third channel of the Rika Denki recorder In the initial phase of an experimental run one-second or two-second intervals ere used. The length of the interval was then increased stepwise up to 40 sec at the end of the run. No correction for coincidence loss was made since the dead time of the internal G-M tube amounted to less than 20 microsec. The voltage characteristics of the G-M tube was regularly checked.

The slowest component of the γ-curv (component IV see below) was constructed from the pparently unexponential decay of the γ-radiation, which was recorded when the β-radiation, registered by the externally placed G-M tube, had reached levels below 2—4 times background activity The alidity of this procedure was tested in the following way In seven experimental runs the seemingly unexponential tail of the γ-curve was recorded for 20—40 min. This part of the elimination curve was then divided into two parts of equal time-length. The steepness of each of the two parts was statistically determined using the method of least squares and compared with each other N statistically significant difference was found ($p > 0.5$)

The wash-out curves recorded by the three detectors were dealt with similarly Background was subtracted and the counts per min were plotted ersus time on semilogarithmic paper Non-linear curves (multiexponential) were analysed by successively subtracting exponentials as described abov (see section A)

The k values of the slopes were calculated according to equation (2) and blood flows according to equation (4) The details of the blood flow determinations will be discussed in subsequent paper (Kampp and Lundgren 1967)

D Local injections of Kr^{85}

Local injections of saline solution containing Kr^{85} were made using microbyrett (Agla micrometer syringe, Burroughs Wellcome and Co London) with thin glass needle (outer diameter 50—200 µm) 5 to 15 µl of the solution, colored by Evan blue, was injected during 30—60 sec into the intestinal wall, either from the serosal side (into the muscularis) or from the mucosal side (into the tissue between the crypts and probably into the submucosa) and also into the perivascular fat of the mesentery T be able to perform the injections from the mucosal side of the intestine, the gut was opened along the antimesenteric border The intestinal segment was then mounted on specially designed metal frame to prevent bleedings from the cut edges Control experiments showed that these operative procedures did not significantly change the microcirculation of the segment (Kampp and Lundgren to be published) After the local injections the needle was withdrawn and the in estinal wall or the mesentery was covered by Mylar The disappearance of the tracer from these local tissue depots was recorded by G-M end window counting tube (Philips N 18504 see sec ion C) oupled to Rika Denki recorder Total enous outflow from the intestine was alway registered by drop-recorder unit as described in section B, except when recordings of the wash-out of Kr^{85} from the perivascular fat were performed.

After sacrificing the animal, attempts were made to precisely localize the dy in the intestinal wall by macroscopic inspection. It proved impossible however to establish the exact site of the injection from such an inspection, often performed 1—3 hrs after the injection, since it could be show that the Evan blue was rapidly transported way in the lymphatic essels. The experiments where local injections in the wall of the gut were made were therefore divided into wo m groups those where the injections were made from the serosal side, into the muscularis-serosa nd those made from the mucosal side into the mucosa-submucosa

Because of the physical propertie of the β-radiation it is possible tha the wash-out curv of Kr^{85} registered by G M tube, might become seriously distorted by diffusion of the tracer towards, or from he detector T test whether such factor had any influence on the r

decay of β activity from a locally injected Kr^{85} depot, the blood flow of the intestine was suddenly stopped by simultaneously clamping the artery and the ein. It was then found that the decline of β-activity eased until the blood flow through the intestine was again restored, proving that the infl enc of the mentioned factor ould be largely neglected.

E. t t adiographic t k q

I a further ttempt t relate ach omponent of the omposite curv to a specific anatomical region, n a toradiogr phic study was performed using antipyrine-C (kindly supplied by professor S. S. Kety). Kr^{85} w not suitable for this part of the present study because of its high-energy β-radiation which blurs the autoradiographic picture and ber use of the technical difficulties incurred in olding ev poration of krypton from thin tissue slices. I series of ontrol experiment it was show that the limination curv of 4-iodoantipyrine-I^{131} had the same gene l appearance as that of Kr^{85}. The utoradiographic technique utilized here was similar to that sed by Kety on the brain (personal communication). About 25 μC antipyrine-C dissol ed in 0.3 0.8 ml saline was administ red into the superior mesenteric artery as slug injection. At predetermined time after the injection, section of the small intestine was extirpated and immediat ly frozen in n acetone and dry ice mixture. The frozen intestinal segme t w sl ed in ryostat (System D ttes Duspn) maintained betwee −70 and −25 C into 5 10 or 70 μm thick transverse sections. The sections were mounted on glass and heated to 65 70 C for bout 30 sec. They were then placed in lose contact w th dental X-ray film (Ilford dental ray film, standard no. 5) and exposed t room temper ture together with a drying agent (silic gel) for arying periods of time (3—45 da) the time dependent on the amount of activity remaining in the tissue.

The localization of ant pyrine-C in the intestinal wall, as indicated by the blackness of the autoradiogr phs, w determined by simultaneous microscopical examination of the histological section nd its corresponding autoradiograph. I orde heck the microscopic observations made the toradiographs w re also densitometrically investigated using a Joyce Lowell mic odensitometre double beam recorde .

A ontrol experimen w re arried out in which the tissue material was kept below −15 C from the in ial freezing to the dev lopment of the exposed ra film. It was clearly demonstrated tha he rapid heating procedure and the subsequent exposure at room temperature in the presence of drying gent did not ause any noticeable diffusion of the tracer in the section. F rthermore was hecked that no blackening of the -ray film took place when it was exposed toghe er with sections from unlabelled intestinal issue.

Results and comments

A The disappe anc of nt a-arterially njected Kr^{85} as egist d by the external scintillation d t tor

Results

The dotted curves in the upper panels of Fig. 1 and 2 illustrate representative elimination curves eg t red after slug injections of an isotonic saline solution containing Kr^{85} into small branch of one of the mesenteric art ries supplying the intestine. These two experiments were performed during constant levels of total intestinal blood flow amounting to 24 ml/min × 100 g in Fig. 1 (resting flow) and to 225 ml/min × 100 g in Fig. 2 (maximal flow) respectively. The ery high intestinal blood flow in Fig. 2 was produced by a constant intra-arterial infusion of isopropylnoradrenaline. The original desaturation curves of the tw o figures can be resolved by successively subtracting exponentials into four components (I– IV) as indicated by the straight lines. The k values of the components, depicting the slopes, are given in the accompanying tables.

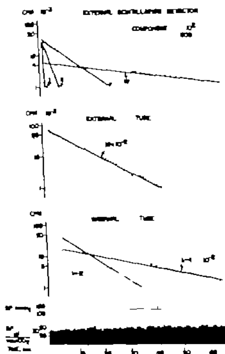


Fig. 1. Cat 2.5 kg. The wash-out of intra-arterially injected Kr^{85} as registered by scintillation detector (upper panel) and by two G-M tubes, one placed outside the intestine the antimesenteric border (second panel) and one located in the intestinal lumen (third panel). The simultaneous registration of mean rterial blood pressure (B. P.) and total intestinal blood flow (B. F.) is shown in the lower panel. The k-values of the elimination curves, depicting their slopes, are given in the Fig. For details, see text.

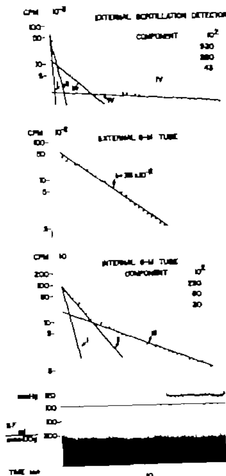


Fig. 2. Cat 3.2 kg. The wash-out of intra-arterially injected Kr^{85} during constant, intra-arterial infusion of isopropylnoradrenaline inducing marked vasodilatation (see lower panel) illustrated in manner similar to that of Fig. 1. For details, see Fig. 1 and text.

A comparison of the corresponding components of Fig. 1 and 2 makes t lea that the k-values for all the four components are greater at the asod lated stat shown in Fig. 2. The difference was particularly pronounced f components II and III.

Comments

It is assumed, when calculating flow rates and flow distribution from the wash-out curves illustrated in the upper panels of Fig. 1 and 2, that the different compartments are parallel-coupled and registered by the detector w th equal efficiency (f

Methods, section A). Furthermore when evaluating blood flow quantitatively an instantaneous diffusion equilibrium between blood and tissue is presumed. There appears to be no anatomical investigation that suggests that there should exist series-coupled vascular beds in the intestinal wall. Moreover the intestinal wall is a symmetrical organ and it seems probable that the different layers were counted with equal efficiency by the external scintillation detector. The activity located in the mesentery may have been registered with lower efficiency in some experiments because of geometrical reasons. The assumption that diffusion equilibrium prevails across the capillary wall will be discussed in a subsequent paper (Kampp and Lundgren 1967).

B. The disappearance of i tra-arterially inje ted Kr^{85} as egistered by Geiger-Müller tubes

In order to localize the different components of the composite γ-curve the elimination of Kr^{85} β-activity from the small intestine was, as mentioned in Methods, also registered by two Geiger-Müller tubes, one located outside the intestine at the antimesenteric border (below called external G-M tube) and one placed in the intestinal lumen (below called internal G-M tube).

1. Exte nal G-M tub

Results

The dotted curves of the second panels of Fig. 1 and illustrate the changes of the β-activity as registered by the external G-M tube simultaneously with the decay of the γ-activity. For geometrical reasons the external G-M tube was mainly recording activity from the serosa and the muscularis of the intestinal wall (see Methods, section C).

At the resting blood flow level in the experiment shown in Fig. 1 a continuous increase in β-activity was observed immediat ly fte the i g injection, and it reached a peak about 1.5 min after the injection. Then a steady decrease of the β-activity ensued which when plotted on semilogarithmic paper formed a straight line as indicated in the figure. Due to uncertainties in recording radioactivity at counts below 50—100 per min, such low ounts were not included in Fig. 1. The above-mentioned, initial increase in β-activity was recorded in several experiments but in no case did it last more than three min in some experiments the decrease of β-activity started almost immediately upon the injection.

A comparison of the two pper panels in Fig. 1 (external scintillation detector and external G-M tube) shows that the k alue of the uniexponential (monoexponential) decrease of β-act ity was of the same order of magnitude as the k value of the third component of the composite γ-curve. A similar agreement is demonstrated in Fig. 2 (maximal asodilatation) between the third component of the γ-curve and the uniexponential portion of the external β-curve (indicated by thin, straight line). This finding held true for all experiments of this study as illustrated in the upper panel of Fig. 3 which summarizes 41 experimental runs,

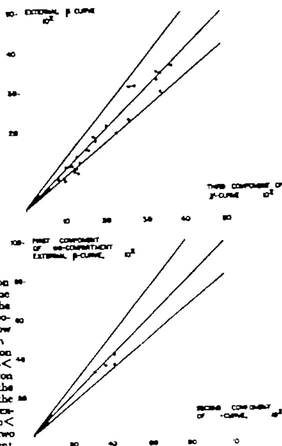


Fig. 3. *Upper panel.* Collected data on the correlation between the k-value of the third component of the γ-curve, on the one hand, and the k-value of unexponential elimination curve or of the slow component of two-component curv registered by the external G-M tube, on the other (= 41 r_{xy} = 0.92 p< 0 001) *Low panel.* Collected data on correlation between the k-value of the second component of the γ-curv and the first component of two-component external β-curv (= 6 r_{xy} = 0.99 p< 0 001) The straight lines in the two diagrams indicate identity ± 20 per cent.

performed t total intestinal blood flows ranging from 15 to 225 ml/min × 100 g tissue. The correlation coefficient (r) was 0 92 (p <0.001) No statistically signif icant difference existed between the two sets of k-values (p >0.8)

In six experimental runs (all except one performed at resting blood flow levels, i.e below 60 ml/min × 100 g) two-component elimination curve was registered by the external G-M tube The k-value of the first component showed good agreement with the second component of the sim ltaneously registered γ-curv as is evident from the lower panel of Fig 3 A high correlation existed between the two k-values (r = 0.99 p <0.001) The two sets of k-values did not differ significantly from each other (p >0.9) The relativ weights of the muscularis in each of these six experimental runs were less than the erage weight, suggesting that the muscularis was unusually thin.

In 1 of the experimental runs in which isopropylnoradrenaline was infused intraarterially to dilate the intestinal ascular bed, an extremely rapid wash-out of activity was observed by the external G-M probe immediately after the injection of the tracer This rapid decrease of radioactiv ty eased within 30 sec and the lev l of

activity remained then more or less constant for 20—40 sec, after which a uni-exponential decay started (see Fig. 2). The upper panel of Fig. 4 illustrates such a desaturation curve recorded in the experiment shown in Fig. 2. (Below this type of elimination of external β-activity will be named initial, rapid decay.)

From Fig. 2 it is evident that a conventional compartmental analysis cannot be performed on the external β-curve. The following analysis was therefore performed on seven runs from three experiments, in which the initial, rapid decay of activity was large enough to be determined with a reasonable degree of accuracy. The level of radioactivity reached after the initial rapid wash-out was regarded as "background" and subtracted from the original elimination curve (see upper panel of Fig. 4). The points thus obtained were plotted on semilogarithmic paper (Fig. 4, lower panel). The k-value was determined from the points, using the method of least squares, and compared with the k-value of the first component of the simultaneously recorded γ-curve. The diagram to the left in Fig. 5 summarizes the results. Considering the crude method of estimating the slope of the initial rapid decay of β-activity the agreement was fairly good between the rapid slopes of the β- and γ-elimination curves. The relative weights of the muscularis in each of these experimental runs were regularly less than the average observed, suggesting that the muscularis was unusually thin.

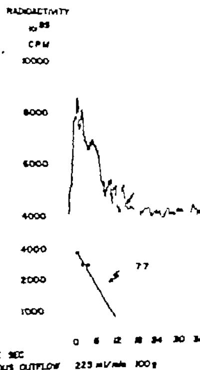


Fig. 4. Cat 3.2 kg. *Upper panel.* The elimination curve registered by the external G-M tube immediately after an intra-arterial injection of Kr^{85}. The experiment, performed during a constant intra-arterial infusion of isopropylnoradrenaline, is the same as that illustrated in Fig. 2. The arrow indicates a change of time constant of the ratemeter from 1 to 3 sec.
Lower panel. The points in the lower panel were deduced by subtracting "background" (indicated by straight line in upper panel) from the original curve. The slope of the curve in the lower panel was deduced from the points by the method of least squares.

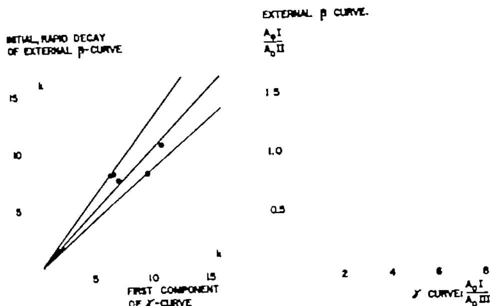


Fig. 5 *Left panel.* Collected data (n = 7) on the correlation between the k-value of the initial, rapid decay of the external β-curve (ordinate) deduced as demonstrated in Fig. 4 and the k-value of the first component of the γ-curve (abscissa). Straight lines denote identity ± 20 per cent.
Right panel. Ratio between the initial activities of the two components of the externally registered β-curve (A_0I/A_0II on ordinate) plotted *versus* the ratio between the initial activities of the first and third components of the γ-curve (A_0I/A_0III on abscissa). All data (n = 6) taken from one and the same experiment (i.e. that of Fig. 2 and 4). r = 0.93 ($p < 0.01$)

The analysis illustrated in the right diagram of Fig. 5 was also performed in order to obtain further evidence for the hypothesis that the initial, rapid decay and the uniexponential terminal wash-out registered by the external β-probe during intestinal vasodilatation corresponded to the first and third components of the γ-curve. In this diagram the ratio between the initial activities of the two components of the externally recorded β-curve (A_0I/A_0II on ordinate) was plotted *versus* the ratio between the initial activities of the first and third components of the γ-curve (A_0I/A_0III on abscissa) all data being taken from one and the same experiment. A good correlation existed between the two ratios (r = 0.93, $p < 0.01$) supporting the proposed hypothesis. It should be pointed out that the second component of the β-curve (A_0II) in the right panel of Fig. 5 was usually the dominating one while the first component of the γ-curve (A_0I) was generally larger than the third component (A_0III). This observation indicates that the second component of the β-curve (corresponding to the third component of the γ-curve) was more closely approximated to the external G-M probe than the first component of the β-curve (corresponding to the first component of the γ-curve).

In no experimental run did the external G-M tube register any wash-out curve with a k-value similar to that of the fourth component of the compound γ-curve

Further no initial, rapid decay of activity was recorded by the external detector in experiments without infusion of the dilator drug not even in animals with a comparatively small weight of the muscularis.

Comments

The results obtained when registering the decrease of β-activity with the external G-M tube strongly suggest that the outer layers of the intestinal wall, i the serosa and the muscularis, were homogenously perfused by blood. This conclusion is based on the observation of a uniexponential elimination curve recorded in the major part of the experimental runs. In the composite —curve the wash-out of Kr^{85} from these outer layers of the intestinal wall seemed to be reflected in the third component (Fig. 3) which showed a k value similar to that of the wash-out curve registered by the external G-M tube

However in 18 experimental runs a two-component disappearance curve was observed. During resting blood flow conditions the k-value of the first component of the β-curve agreed well with the k value of the second component of the simultaneously registered —curv (Fig 3 lower panel) On the other hand, when vasodilatation was induced. the slope of the initial rapid decay of β-activity seemed to coincide with the slope of the first component of the -curv (Fig. 4 and 5 It should be stressed that the relative weight of the muscularis was regularly less than average in these experiment and hence the muscularis was presumably comparatively thin. Thus some β-activity f om g the submucosa was probably also registered by the external C M tube under such circumstances. Since the slow components of the two-component β-curves nearly alwa ere the dominating es (cf Fig 5 right panel it is concluded that the first components were localized one of the inner la er of the intestinal wall. g the submucosa.

It should be pointed out in this connection that a fast initial component of considerable magnitude was present in the -curv also at resting blood flows (Fig. 1) Any initial apid dec of activity of the kind illustrated in Fig. 4 was, however never recorded by the external G-M tube at resting blood flows. This observation seems to indic t that at least parts of the fastest component of the γ-curve was localized in different portions of the intestinal wall during resting" flow and during vasodilatation Since the external G-M tube only seemed to register the first component of the omposite -curv when the intestinal vascular bed was dilated, it seems reasonable to onclude that the localization of the first component then was closer to the external G-M tube than at resting blood flows.

2 Internal G-M tub

Results

The dotted curves in the third panel from the top of Fig 1 (resting blood flow 24 ml/min $\times$ 100 g) and Fig. 2 (vasodilatation, 225 ml/min $\times$ 100 g) illustrate the changes in radioactivity registered by a G-M tube placed in the intestinal lumen. In both these figures a prolonged increase in recorded radioactivity was observed

after the intra-arterial injection before the gradual disappearance of activity. A difference in time characteristics between the two curves of Fig. 1 and 2 is, however noted. When "resting" flow conditions prevailed (Fig. 1) the decrease of radioactivity started about 3 min after the slug injection of Kr^{85} while during intense vasodilatation (Fig. 2) the elimination of Kr^{85} began after only 20 sec.

The curve relating the magnitude of venous outflow to the average time lag between the injection and the start of the decrease of activity ("time of delay") as registered by the internal G-M tube, is shown in Fig. 6 (●————●). Below a venous outflow of 50—60 ml/min × 100 g the time of delay was found to be clearly flow-dependent, while above that blood flow level the time lag seemed to be largely independent of flow. For the sake of comparison the corresponding curve for the external G-M tube (○— — — —○) is also included in Fig. 6 (*cf.* the second panel of *Fig. 1*). *It is seen that there was no relation between the magnitude of blood* flow and the time of delay in recordings with the external G-M probe.

The appearance of the elimination curve, recorded by the internal G-M probe, varied considerably with the level of blood flow. A *uniexponential disappearance* curve was usually registered at low blood flows. Fig. 1 represents in this respect an exception from the rule, since here the elimination curve can be resolved into two components. In most experimental runs performed at somewhat raised levels of blood flow between 50 and 150 ml/min × 100 g, a two-component curve was recorded while a three-component curve was always observed when venous outflow exceeded 150 ml/min × 100 g (see Fig. 2).

A comparison between the k-values of the different components of the internal β-curve and the k-values of the four components of the simultaneously registered γ-curve failed to reveal any consistent relationship of the kind illustrated in Fig. 3 with one exception. The fastest component of the internal β-curve agreed fairly well with the second *phase of the γ-curve when venous outflow exceeded 150 ml/min* × 100 g (*cf.* Fig. 2). At blood flow levels between 50 and 150 ml/min × 100 g the k-value of the fastest of the two components of the internal β-curve fell between k-values of the second and third components of the γ-curve. The k-value of the uniexponential curve registered by the internal detector at low levels of blood flow was less than the k-value of the third component of the γ-curve but usually somewhat larger than that of the fourth. In no experiment did the internal G-M tube register any fast, initial decay curve of the kind recorded by the external G-M tube (Fig. 4).

Comment

The radioactivity registered by a G-M tube placed in the intestinal lumen after an intra-arterial injection of Kr^{85} emanated mainly from tracer present in the villi and in the lumen of the gut. Consequently desaturation curves recorded by such a detector may reflect one or both of two different physiological phenomena, *i.e.* a "true" vascular wash-out of Kr^{85} from the intestinal wall and an absorption of the tracer across the intestinal epithelium. No method exists which makes it possible

to differentiate between these two events in an elimination curve. It seems, therefore, impossible to draw any conclusions concerning the blood flow of the mucosa from the results obtained with the internal G-M tube.

Yet another type of mechanism, which distorted the "true" vascular wash-out curve registered by the internal β-probe, was in all probability present in the lower range of total intestinal blood flow. Evidence has been presented which suggests that a countercurrent exchange of easily diffusible substances, such as oxygen and antipyrine takes place between the ascending and descending limbs of the mucosal vascular loops, located mainly in the villi (Kampp and Lundgren 1966 b, Kampp, Lundgren and Nilsson 1967). Such a mechanism tends to retard the vascular elimination of substances "trapped" in the vascular loop. The very slow rate of elimination of Kr^{85} recorded by the internal G-M probe at low intestinal blood flows probably reflected such a delayed wash-out (*cf.* Thorburn *et al.* 1963, Aukland and Berliner 1964). The data presented in Fig. 6 also seem to be consistent with the occurrence of a countercurrent exchange in the mucosa. The considerable time lag between the injection and the start of the desaturation of activity recorded by the internal detector may be explained in the following way. Immediately after the slug injection of Kr^{85} there existed a concentration gradient of the isotope within the vascular loops of the villi, the highest concentration of the tracer being located between the crypts and at the bases of the villi. However, the vascular loop arrangement tended to establish a reverse gradient, which brought about a transport of Kr^{85} from the bases towards the tips of the villi, a relatively slow movement of the isotope towards the internal detector. This gradual increase of Kr^{85} concentration in the vicinity of the internal G-M tube was recorded by this detector as a continuous increase in radioactivity masking the simultaneous wash-out of Kr^{85}. When blood flow was increased mean transit time of blood in the vascular loops of the villi grew shorter. Finally a blood flow level was reached at which transit time became

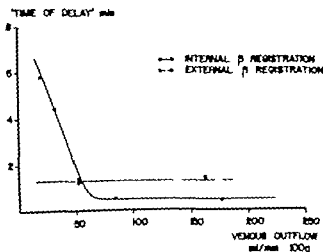


Fig. 6. The correlation between the time of delay (= the time lag between the injection of the detracer and the start of the decrease of β-activity as registered by the external and internal G-M tubes) and recorded total intestinal blood flow. Dots denote means of paired values. Lines drawn by inspection. Vasodilatation was induced by constant, intra-arterial infusions of isopropylnoradrenaline. For details, see text.

too short to allow for any significant countercurrent exchange of Kr85 To judge from the data presented in Fig. 6 this situation was reached when total intestinal blood flow exceeded 70 ml/min × 100 g The "time of delay" recorded above that flow level in all probability represents a diffusion of the tracer towards the probe Such an explanation seems also probable for the flow-independent time lag recorded by the external probe (Fig 6 dotted line)

C. Local injections of Kr85

Results

In four experiments minute amounts of a solution containing Kr85 (5—15 µl) were injected into the perivascular fat of the mesentery The decrease in radioactivity *versus* time was plotted on semilogarithmic paper and formed a straight line even in experimental runs prolonged for 60—90 min. The k value of the slope (mean value 0.036 range 0.012—0.057 n = 10) was of the same order of magnitude as that of the fourth component of the γ-curve Intestinal blood flow was not directly registered in these experiments, performed under "resting" conditions.

Injections of a small volume of the Kr85 solution were also made into the intestinal wall from the mucosal (3 expts.) and from the serosal side (6 expts.) at various levels of total intestinal blood flow The desaturation curves registered after each injection formed a straight line when plotted on semilogarithmic paper even in such runs where the registration was continued until an activity level around 5 per cent of the initial one was reached. The slope of the curves, expressed in terms of blood flow using equation (4) (see Methods, section A) was compared with the blood flow values of the different components of the composite γ-curve The result are summarized in Fig. 7 (dots) in which the injections made from the mucosal side

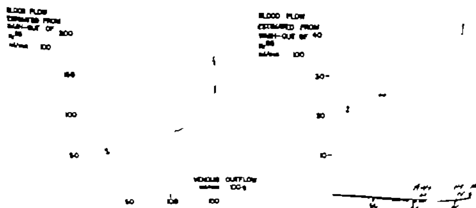


Fig 7 Comparisons between the slopes of second and third components of the composite γ-curve and the slopes of the monoexponential elimination curves registered after injections performed from the mucosal side (left panel) and the serosal side (right panel). Dots denote local injections. Shaded areas indicate the dispersion of data ... values of the second (left panel) and third (right panel) components ... intestinal blood flow (from Kampp and Lundgren 1967)

are plotted *versus* recorded venous outflow in the left panel (n = 19) while the injections made from the serosal side are plotted in a similar manner in the right panel (n = 24). The shaded areas of the two diagrams indicate the dispersion of data relating the blood flow values of the second and third components of the γ-curve respectively to venous outflow (see Kampp and Lundgren 1967). It can be seen that most observations fall within the shaded areas.

Comments

The results described above obtained after local injections of the tracer into different parts of the intestinal preparation, indicate that the wash-out of Kr^{85} from the perivascular fat of the mesentery was reflected in the fourth component of the γ-curve. Furthermore the results suggest that the elimination from the seromuscularis was reflected in the third component of the γ-curve and the disappearance from the mucosa-submucosa in the second component of the γ-curve.

D. The localization of intra-arterially injected antipyrine-C^{14} as revealed by autoradiography

Results

Fig. 8 and 9 illustrate representative autoradiographs of intestinal segments removed at various times after intra-arterial injections of antipyrine-C^{14}. The corresponding histological sections are also included in the figures. The experiment of Fig. 8 was performed at a venous outflow level of 21 ml/min × 100 g, while the experiment of Fig. 9 was made during a constant close intra-arterial infusion of isopropylnoradrenaline inducing a vasodilatation amounting to about 100 ml/min × 100 g.

Segment A of Fig. 8 was removed about five sec after the injection. Most of radioactivity was then localized between the crypts and at the bases of the villi, to a minor extent also in the submucosa when judged by the blackening of the autoradiographs. A comparatively small amount of activity was observed in the villi while the circular layer of the muscularis was largely devoid of blackening. The concentration of the tracer in longitudinal layer of the muscularis seemed often higher than in the circular layers, although areas with equal blackness were observed regularly in each cross-section. The large veins, located just beneath the serosa, were easily discernible on microscopic examination of the autoradiographs as round structures.

The intestinal segment of section B of Fig. 8 was removed 3.0 min after the injection. It is evident from this figure that the relative distribution of the antipyrine-C^{14} remaining in the intestinal wall differed markedly that of section A of Fig. 8. Thus, the concentration of antipyrine-C seemed to be roughly equal throughout the intestinal wall.

It seems reasonable to conclude when comparing sections A and B of Fig. 8 that the antipyrine-C^{14} washed out from the intestinal wall by the blood during the first three min after an intra-arterial injection, mainly emanated from the bases of the villi, from the crypts and adjacent parts of the submucosa. This observation suggests



Fig. 8 Cat 2.8 kg. Autoradiographs (right) and corresponding histological sections (left) of three intestinal segments removed at various times after an intra-arterial injection of antipyrine-C¹⁴. Recorded total intestinal blood flow amounted to 21 ml/min × 100 g. Exposure times of autoradiographs 5 sec segment 3 days, 3 min segment 11 days, 13 min 30 sec segment 19 days

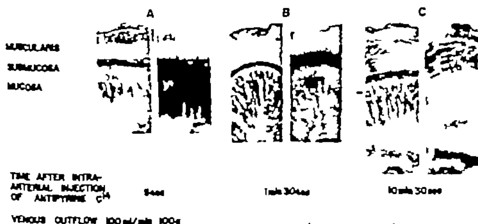


Fig. 9 Cat 2.3 kg. Autoradiographs (right) and corresponding histological sections (left) of three intestinal segments removed at various times after an intra-arterial injection of antipyrine-C¹⁴. The experiment was performed during a constant, intra-arterial infusion of isopropylnoradrenaline inducing vasodilatation amounting to 100 ml/min × 100 g. Exposure times of autoradiographs 5 sec segment 3 days, 1 min 30 sec segment 32 days, 10 min 30 sec segment 43 days.

that the elimination rates of antipyrine-C¹⁴ from these parts of the intestinal wall was faster than in the villi and the muscularis, a conclusion which is further strengthened by the appearance of section C of Fig. 8. The intestinal segment illustrated in that section was removed 13.5 min after injection. The activity then resided mainly in the muscularis and in the tips of the villi.

The intestinal segment illustrated in section A of Fig. 9 was extirpated during an induced vasodilatation about five sec after the intra-arterial injection

tipyrine-C^{14} i.e. the time of removal was the same as for the corresponding section of Fig. 8. The appearance of the autoradiograph of section A of Fig. 9 differs in two respects from that of section A of Fig. 8. Firstly a considerable amount of antipyrine-C^{14} seems to have reached the villi to judge by the heavy blackening of these structures. Secondly the submucosa seems to contain comparatively more antipyrine-C^{14}. In the circular layer of the muscularis there was little or no blackening while the longitudinal portion was blackened in certain areas (*cf.* section A of Fig. 8).

The segment shown in section B of Fig. 9 was removed 1.5 min after the injection of the tracer. It is noted that the two black bands of section 9 A are absent in this second section. In fact a light band could now be seen in the submucosa and in adjacent tissues between the crypts. Such a phenomenon was never noted in experiments performed at "resting" outflow levels. These observations suggest that the elimination rate of antipyrine-C^{14} from the tips of the villi and from the submucosa and adjacent parts of the mucosa was very rapid.

Section C of Fig. 9 illustrates the appearance of the autoradiograph of an intestinal segment removed about 10.5 min after the injection of antipyrine. The activity remaining in the tissue was here located in the muscularis and, to a small extent, also in the tips of the villi (*cf.* section C of Fig. 8).

It is evident from Fig. 8 as well as from Fig. 9 that the relative amount of the tracer in the lumen of the intestine was largest in the segments extirpated 10—14 min after the injection (compare sections A and C of both figures). This observation indicates that at least part of the antipyrine-C^{14} located in the lumen of the gut was washed out slowly. This conclusion is further strengthened by the appearance in the autoradiographs of the intestinal segments removed 30—40 min after the intra-arterial injection of the tracer (not shown in the figures). At that time the concentration of antipyrine-C^{14} in the muscularis was definitely less than that of the lumen.

When examining the autoradiographs at a higher magnification than illustrated in Fig. 8 and 9, submucosal areas of blackening equal to that of the muscularis were observed in segments removed 10–14 min after the injection.

The observations described above were further checked in two ways. First, by a careful microscopic examination at a higher magnification than that illustrated in Fig. 8 and 9 and second by densitometric investigation (see Methods, section E).

Comments

Antipyrine and krypton are non-metabolized lipid soluble substances and their molecular weight are of the same order of magnitude. It seems therefore reasonable to assume that the kinetics of the intra-arterially injected antipyrine-C^{14} is similar to that of Kr^{85} administered in an identical manner at least during the comparatively short experimental runs of the present study. This view was corroborated by the finding of similar wash-out curves from the intestine for the two tracers after intra-arterial injection. It could, however, be argued that the antipyrine, in contrast to krypton, was not eliminated when passing the lungs, and hence, the arterial

concentration was not negligibly low for this tracer. Since the injected antipyrine was diluted in the volume of total body water after leaving the intestine, the concentration of this solute in the arterial blood supplying the intestine must have been low. Furthermore, only one experimental run was made on each animal. Thus, it seems justified to compare the results obtained in the autoradiographic study with those of the experiments using Kr^{85} described above.

During the initial 1—3 min of an experimental run most of the Kr^{85} leaving the intestine emanated from the first component (see Fig. 1 and 2). From a comparison between section A and B of Fig. 8 and 9 it would therefore be possible to localize the first component. When "resting" intestinal blood flow prevailed, the highest rate of elimination of antipyrine-C seemed to occur from the tissue between the crypts and from the bases of the villi (Fig. 8) while at increased intestinal blood flow the elimination of the tracer from the submucosa and adjacent mucosal parts was extremely fast (Fig. 9). These observations seem to corroborate the conclusions drawn from the recordings of the external G-M tube (Results, section B 1). In a subsequent paper evidence will be presented which suggests that the fast wash-out from the tips of the villi, illustrated in Fig. 9 reflected a countercurrent exchange of the tracer (Kampp, Lundgren and Sjöstrand, to be published).

The radioactivity eliminated from the intestine between sections B and C of Fig. 8 and 9 mainly stems from the second component (see Fig. 1 and 2). Comparing these two autoradiographs one may conclude that the second component is localized in the mucosa and submucosa in agreement with the findings obtained with the external G-M tube (Fig. 3 lower panel) and with the local injections (Fig. 7 left panel).

The appearance of the autoradiographs of the intestinal segments removed 8—14 min after the injection (sections C of Fig. 8 and 9) indicate that the rate of wash-out of the tracer from the muscularis and from the intestinal lumen was considerably slower than from most parts of the submucosa and the mucosa. The elimination rate from certain parts of the submucosa and from the tips of the villi, particularly at "resting" blood flow (Fig. 8) seems to have been of the same order of magnitude as that of the muscularis and the intestinal lumen. Since the autoradiographs, performed on intestinal segments removed 30—43 min after the injection, showed that the activity remaining in the cross-sections was localized predominantly to the intestinal lumen and in the tips of the villi, it is concluded that a major part of the third component emanated from the muscularis and to a small extent also from the submucosa (cf. Results, section B 1 and E). Furthermore one may infer that the wash-out of antipyrine-C from the lumen and the tips of the villi occurred at a very slow rate (cf. Results, section B 2 and E).

E. Comparisons between calculated and measured relative tissue weights

Results

The relative weights of the four compartments were calculated in the following way. When venous outflow amounted to a value less than 60 ml/min × 100 g the weight

percentage was calculated from the deduced data on the amount of tracer initially distributed to the different compartments, their calculated blood flows and from the recorded venous outflow It was believed that the γ-curve did not exclusively reflect vascular events below a venous outflow level of 60 ml/min / 100 g, but also in all probability a countercurrent exchange of the tracer in the mucosa. Hence, the flow as calculated from the γ-curve will in this situation not exactly reflect the true intestinal blood flow When, on the other hand, the directly recorded total blood flow exceeded 60 ml/min × 100 g, calculations could more reliably be based on flow values as computed from the γ-curve since the countercurrent exchange was small or even negligible

The estimated relative weight of the third compartment was compared with the measured relative weight of the muscularis. The third compartment was found to correspond to a somewhat larger portion of the intestine than the muscularis in most experiments, the ratio between them being usually between 1.0—1.5 independent of the magnitude of venous outflow The mean value amounted to 1 19.

The relative weight of the fourth compartment showed a declining trend with flow Below a venous outflow of 100 ml/min × 100 g the average relative weight of this compartment amounted to 17—19 per cent while it decreased continuously above that flow level to about 9 per cent at maximum dilatation. In 24 experimental runs, the weight of the mesentery and its vessels was determined and, thus, the relative weight of this tissue could be calculated in per cent of the total weight of the intestine and its mesentery Most observations fell between 8 and 14 per cent.

omment

he results described in sections B 1 C and D indicate that the muscularis and the erosa were fairly homogenously perfused, and that their blood flow was reflected n the third component of the γ-curve The comparison of relative weights of the third compartment and the muscularis suggests that the rate of elimination of Kr^{85} from another part of the intestinal preparation may equal that of the third component. This conclusion was corroborated by the autoradiographic observation that the wash-out of antipyrine-C from submucosal parts occurred at a rate similar to that of the third component (Results, section D)

The calculated relative weight of the fourth compartment was larger than the measured relative weight of the mesentery when the total blood flow of the intestinal preparation was below 100 ml/min × 100 g but of largely the same magnitude when the total blood flow was above 100 ml/min × 100 g Since the mesentery includes other tissues than perivascular fat it is concluded that the wash-out of Kr^{85} from some other part of the intestinal preparation e.g from the intestinal lumen (cf. Results, sections B 2 and D) was also mirrored in the slowest component.

General discussion

Any wash-out curve which, when plotted on semilogarithmic paper has a general appearance similar to that illustrated in the upper panels of Fig 1 and 2, can be resolved into a number of exponential functions. However the mere fact that such

an analysis can be performed does not necessarily imply that the different components have any physiological significance. One may for example by this analysis mainly categorize a continuum of k-values (*cf.* Dobson and Warner 1957 Briscoe and Cournand 1959 Kjellmer *et al.* 1967)

In the present series of experiments, the registration of the elimination of γ-activity was complemented by four independent methods (see Results, sections B C, D and E) This was made in an attempt to establish whether the four components of the γ-curve had any physiological significance, in the sense that they were localized to particular parts of the intestinal preparation. It was *a priori* suspected that so was the case, since the intestinal preparation contains some well-defined anatomical sub-units such as the muscularis, mucosa and submucosa. An attempt will here be made to present a summarizing picture of the results obtained with the different techniques.

Component I The composite γ-curve exhibited an extremely fast initial component at all venous outflow levels. However some observations in the present study suggested that this component was located in different parts of the intestinal wall at low and high intestinal blood flows, respectively Thus, the external G-M tube seemed to register the first component only during induced vasodilatation, and then only in cats with a comparatively thin intestinal muscularis (Results, section B 1) Further the different appearance of the autoradiographs (Results, section D) at resting" flow and during vasodilatation also indicated different localizations of the first component of the γ-curve.

Evidence has recently been presented which suggests that a countercurrent exchange of easily diffusible substances such as oxygen and 4-iodoantipyrine takes place between the ascending and descending limbs of the mucosal vascular loops, chiefly located in the villi (Kampp and Lundgren 1966 b Kampp, Lundgren and Nilsson 1967) It therefore seems probable that intra-arterially injected krypton was shunted in the countercurrent exchanger in a manner similar to oxygen and 4-iodoantipyrine. It is accordingly proposed that the initial rapid component of the composite γ-curve predominantly reflected such a mechanism at low or moderate intestinal blood flow levels. The relative absence of antipyrine-C[14] in the villi at the venous outflow level of Fig. 8 is in accordance with the proposed hypothesis, since the countercurrent exchanger will then act as a barrier for the net transport of easily diffusible substances along the long axes of the vessels (*cf.* Thorburn *et al.* 1963) Furthermore, the slow wash-out from the tips of the villi observed at low and moderate flow levels (Results, sections B.2 and D) also confirms the hypothesis so does the results illustrated in Fig. 6.

As blood flow through the intestine was increased, the efficiency of the countercurrent exchanger in the villi appeared to decrease (*cf.* Results section B 2 and Fig. 6 see also Fig. 3 in Kampp and Lundgren 1966) and the first very rapid component now reflected more and more the blood flow through an extremely well-perfused region that was probably located in the mucosa and the submucosa. This opinion is based on the results obtained by the external G-M tube (Results, section

percentage was calculated from the deduced data on the amount of tracer initially distributed to the different compartments, their calculated blood flows and from the recorded venous outflow. It was believed that the γ-curve did not exclusively reflect vascular events below a venous outflow level of 60 ml/min × 100 g, but also in all probability a countercurrent exchange of the tracer in the mucosa. Hence, the flow as calculated from the γ-curve will in this situation not exactly reflect the true intestinal blood flow. When, on the other hand, the directly recorded total blood flow exceeded 60 ml/min × 100 g, calculations could more reliably be based on flow values as computed from the γ-curve since the countercurrent exchange was small or even negligible.

The estimated relative weight of the third compartment was compared with the measured relative weight of the muscularis. The third compartment was found to correspond to a somewhat larger portion of the intestine than the muscularis in most experiments, the ratio between them being usually between 1.0—1.5, independent of the magnitude of venous outflow. The mean value amounted to 1.19.

The relative weight of the fourth compartment showed a declining trend with flow. Below a venous outflow of 100 ml/min × 100 g the average relative weight of this compartment amounted to 17—19 per cent while it decreased continuously above that flow level to about 9 per cent at maximum dilatation. In 24 experimental runs, the weight of the mesentery and its vessels was determined and, thus, the relative weight of this tissue could be calculated in per cent of the total weight of the intestine and its mesentery. Most observations fell between 8 and 14 per cent.

omments

he results described in sections B 1, C and D indicate that the muscularis and the erosa were fairly homogenously perfused, and that their blood flow was reflected in the third component of the γ-curve. The comparison of relative weights of the third compartment and the muscularis suggests that the rate of limmation of Kr^{85} from another part of the intestinal preparation may equal that of the third component. This conclusion was corroborated by the autoradiographic observation that the wash-out of antipyrine-C from submucosal parts occurred at a rate similar to that of the third component (Results, section D).

The calculated relative weight of the fourth compartment was larger than the measured relative weight of the mesentery when the total blood flow of the intestinal preparation was below 100 ml/min × 100 g but of largely the same magnitude when the total blood flow was bove 100 ml/min × 100 g. Since the mesentery includes other tissues than perivascular fat, it is concluded that the wash-out of Kr^{85} from some other part of the intestinal preparation, e.g. from the intestinal lumen (cf. Results, sections B 2 and D) was also mirrored in the slowest component.

General discussion

Any wash-out curve which, when plotted on semilogarithmic paper has a general appearance similar to that illustrated n the pper panels of Fig. 1 and 2, can be resolved into a number of exponential functions. However the mere fact that such

D) and possibly also by the multiexponential elimination curve recorded by the internal G-M tube (see Fig. 1 and 2)

Component IV The local injections (Results, section C) provided evidence that the rate of elimination of Kr[85] from the perivascular fat of the mesentery was of the same order of magnitude as that of the fourth component. Furthermore, the results obtained with the autoradiographic technique (section D) suggested that an absorption of the tracer from the intestinal lumen was to some extent also reflected in this component, an interpretation which was confirmed by the rate of wash-out registered by the internal G-M tube (section B.2) particularly at low venous outflow levels (Fig. 1)

When trying to determine the flow rates and the flow distribution within the intestine by analysing the wash-out curve of intra arterially injected Kr[85] the presence of a countercurrent exchange of the tracer in the mucosa in the lower flow range complicated the analysis. This mechanism was not only reflected in a fast, initial component of the elimination curve but may also have distorted the rest of the curve since a certain amount of the tracer might have been "trapped" in the villous loops and delayed in its wash-out (*cf* Thorburn *et al.* 1963 Aukland and Berliner 1964) It seems reasonable to assume that the eventual distortion mainly affected the slowest components (III, IV) since the flow in the villi in all probability was as great as that of the second compartment (see above)

The possible distortion of the wash-out curve seems not to have affected the k-values of the second and third components, to judge by the agreement between the slopes of these components of the γ-curve and the k values of the external β-curve (Fig. 3) Since the external G-M probe did not register radioactivity in the villi, a difference in k-values would have been noted, had the slope of the second or third component been significantly distorted.

The comparisons of relative weights, performed in section E of the Results, seem to offer a possibility to evaluate the effect on the distribution data of the four components of the activity "trapped" in the villous loops. The observation that the relative weight of the third compartment remained roughly constant independent of flow seems to exclude that any major portion of the delayed wash-out of Kr[85] from the villous vascular loops was reflected in this component. Had that been the case, the relative weight of the third compartment should have decreased as the countercurrent exchanger grew less efficient with increasing flow (*cf* Fig 6) Instead, the weight analysis indicated that the size of the slowest compartment (IV) decreased as blood flow was augmented. The decrease in size coincided fairly well with the decreased efficiency of the countercurrent exchanger with increased flow (*cf* Fig. 6 in the present paper and Fig. 3 in Kampp and Lundgren 1967) This observation suggests, accordingly that the delayed elimination from the vascular loops of the villi was mainly reflected in the fourth component of the γ-curve

This work was supported by grants from the Swedish Medical Research Council (B67 14X-1631B), from Air Force School of Aerospace Research (OAR) United States Air Force under Contract AF 61(052) 732, from U S. Public Health Service (HE-05675-06) and from the Faculty of Medicine, University of Göteborg.

References

AUKLAND, K. and R. W. BERLINER, Renal medullary count rcurrent system studied with hydrogen gas. *Circulat. Res.* 1964. *1* 430—442.

AUKLAND, K., B. F. BOWER and R. W. BERLINER, Measurement of local blood flow with hydrogen gas. *Circulat. Res.* 1964. *14* 164—187.

BARCROFT, B. P. *S e ctory m hanism of th dig stu gla ds.* Ed. 2. Paul B. Hoeber Inc. New York 1950.

BRISCOE, W. A. and A. COURNAND, Uneven ntilation of normal and diseased lungs studied by an open-circuit method. *J. appl. Physiol.* 1959. *14* 84—290.

CALABRAY, L., F. WOLF and V. VARRO, Der Kreislaufgradient im Dünndarm. *Z. Gastroenterologie* 1965. *3* 61—65.

DOBSON, E. L. and G. F. WARNER, Measurement of regional sodium turnover rates and their pplication to the estimation of regional blood flow. *Am. J. Physiol.* 1957. *189* 269—272.

DRESEL, P., B. FOLKOW and I. WALLENTIN, Rubidium[86] clearanc during neurogenic redistribution of intestinal blood flow. *Act. physiol. cand.* 1966. *67* 173—184.

FOLKOW, B. Regional adj stments of intestinal blood flow. *Gastroenterology* 1967. *52* 423—432.

FOLKOW, B., O. LUNDGREN and I. WALLENTIN, Studies on the relationship between flow resistance, pillary filt tion coefficient and regional blood olume in the intestine of the cat. *Act. phy iol sc nd.* 1963. *57* 2 0—283.

GRAYSON, J. and D. MENDEL, *Phy iology of th splanchnic circulation.* Monographs of the Physiological Society No. 14. London, Edward Arnold Ltd. 1965.

GRIM, E. *The flow of blood th mesent c sels.* In Handbook of Physiol. 1963. Section 2 (Circulation). Vol. II. 1439—1456.

GRIM, E. and E. O. LINDSETH, Distribution of blood flow to the tissues of the small intestine of the dog. *U M J.* 1958. *30* 138—145.

HAGGENDAL, E., N. J. NILSSON and B. NORBÄCK, On the components of Kr[85] clearance curves from the brain of the dog. *Acta physiol. scand.* 1965. *66* Suppl. 258. 5—25.

HERD, J. A., M. HOLLENBERG, G. D. THORBURN, H. H. KOPALD and A. C. BARGER, Myocardial blood flow determined with krypton[85] in unanesthetized dogs. *Amer. J. Physiol.* 1962. *203* 122—124.

INGVAR, D. H. and N. A. LASSEN, Regional blood flow of the erebral ortex determined by krypton[85]. *Act. physiol. sc nd.* 196 . *54* 3 5—338.

JANSSON, G., M. KAMPP, O. LUNDGREN and J. MARTINSSON, Studies on the circulation of the stomach. *Act. physiol. sc nd.* 1966. *68* Suppl. 77. 91.

JONES, H. B. Respiratory system: Nitrogen limination. *I.* Glasser O. *Medical Physic* 1950. 855—871.

KAMPP, M. and O. LUNDGREN, Blood flow and flow distribution within the small intestine of the . *Acta physiol. scand.* 1966 a. *68* Suppl. 277. 102.

KAMPP, M. and O. LUNDGREN, E idenc for countercurrent exchange in intestinal villi. *Acta physiol. scand.* 1966 b. *68* Suppl. 277. 103.

KAMPP, M. and O. LUNDGREN, Blood flow and flow distribution in the small intestine of the t as analysed by the Kr[85] ash-ou technique. *Acta physiol. scand.* 1968. *72* 282—297.

KAMPP, M., O. LUNDGREN and N. J. NILSSON, Extravascular short-circuiting of oxygen indicating countercurrent exchange in the intestinal illi of the cat. *Experientia* (Basel) 1967. *23* 197—198.

KETY, S. S. The theory nd pplications of the exchange of inert gas at the lungs and tissues. *Pharmacol. Rev.* 1951. *3* 1—41.

KETY, S. S. Theory of blood tissue exchange and its application to measurement of blood flow. *Meth. med. Res.* 1960. *8* 223—227.

KETY, S. S. Measurement of local circulation within the brain by means of inert, diffusible tracers: examination of the theory, assumptions and possible sources of error. *Acta neurol. scand.* 1965. Suppl. 14. 9—12.

KJELLMER, I., I. LINDBJERG, I. PREROVSKY and H. TONNESEN, The relation between blood flow in an isolated muscle measured with the Xe[133] clearance and direct recording technique. *Act. physiol. and.* 1967. *69* 69—78.

LADEFOGED, J., F. PEDERSEN, L. DOUTHEIL, P. DEETJEN and E. E. SELKURT, Renal blood flow measured with xenon[133] wash-out technique and with an electromagnetic flowmeter. *Pflüg. A. h. ges. Physiol.* 1965. *284* 195—200.

LANDIS, E. M. and J. R. PAPPENHEIMER, *Ex ha g of substances through the c pillary walls.* In Handbook of Physiol. 1963. Section 2 (Circulation). Vol. II. 961—1034.

LARSEN, O. A., N. A. LASSEN and F. QUAADE, Blood flow through human adipose tissue determined with radioactive xenon. *Acta phy iol. cand.* 1966. *66* 337—345.

LASSEN, N. A., Muscle blood flow in normal man and in patients with intermittent claudication evaluated by simultaneous Xe^{133} and Na^{24} clearances. *J. clin. Invest.* 1964. *43*. 1805—1812.

LINDER, E. Measurements of normal and collateral coronary blood flow by close-arterial and intramyocardial injection of $Krypton^{85}$ and $Xenon^{133}$. *Acta physiol. scand.* 1966. *68* Suppl. 272. 5—31.

LINDGREN, P. An improved method for drop recording of arterial or venous blood flow. *Acta physiol. scand.* 1958. *42*. 5—11.

LUNDGREN, O. and M. KAMPP. The wash-out of intraarterially injected $krypton^{85}$ from the intestine of the cat. *Experientia* (Basel) 1966. *22*. 268—270.

LUNDGREN, O. and S. MELLANDER, Augmentation of tissue-blood transfer of solutes by transcapillary filtration and absorption. *Acta physiol. scand.* 1967. *70*. 26—41.

PITTS, R. F. Discussion of "The indicator fractionation technique for the study of regional blood flow". *Gastroenterology* 1967. *52*. 371—373.

RAYNER, R., L. MACLEAN and E. GRIM, Intestinal tissue blood flow in shock due to endotoxin. *Circulat. Res.* 1960. *8*. 1212—1217.

RENKIN, E. M. Transport of $potassium^{42}$ from blood to tissue in isolated mammalian skeletal muscle. *Amer. J. Physiol.* 1959. *197*. 1205—1215.

ROSS, R. S., K. UEDA, P. R. LICHTLEN and J. R. REES, Measurement of myocardial blood flow in animals and man by selective injection of radioactive inert gas into the coronary arteries. *Circulat. Res.* 1964. *15*. 28—41.

SPANNER, R., Neue Befunde über die Blutwege der Darmwand und ihre funktionelle Bedeutung. *Morph. Jb.* 1932. *69*. 394—454.

THORBURN, G. D., B. H. CAREY and G. S. MOLYNEUX, Distribution of blood flow within the skin of the rabbit with particular references to hair growth. *Circulat. Res.* 1966. *18*. 650—659.

THORBURN, G. D., H. H. KOPALD, J. A. HERD, M. HOLLENBERG, C. C. C. O'MORCHOE and A. C. BARGER, Intrarenal distribution of nutrient blood flow determined with $krypton^{85}$ in the unanesthetized dog. *Circulat. Res.* 1963. *13*. 290—307.

WINNE, D. *Kinetics of distribution of D_2O in the tissue of the canine ileum* (Thesis). Minneapolis Univ. of Minnesota. 1961. Quoted from Grim 1963.

WINNE, D. E. and E. GRIM, Kinetics of distribution of D_2O in canine intestinal tissues. *Amer. J. Physiol.* 1966. *211*. 600—606.

Acta physiol. scand. 1968. 72. 282—297

From the Department of Physiology University of Goteborg, Sweden

Blood Flow and Flow Distribution in the Small Intestine of the Cat as Analysed by the Kr ⁸ Wash Out Technique

By

MOGENS KAMPP and OVE LUNDGREN

Received 23 J ne 1967

Abstract

KAMPP M and O LUNDGREN *Blood flow and flow distrib tion in the small in-te tine f the cat as analysed by the Kr⁸ wash-out tech ique* Acta physiol. scand. 1968. 72 282—297

The blood flow and flow distribution of the small intestine of t w e studied at arious levels of ascular smooth muscle tone by means of the Kr^{85} wash-out technique. At "resting" total intestinal blood flow about 80 per cent of flow was distributed to the mucosa-submucosa, where the flow rate amou ted to 40—80 ml/min × 100 g. Blood flow of the muscularis as then 10—20 ml/mi × 100 g. As total intestinal blood flow was increased by constant, intra-arterial infusions of isopropylnoradrenaline larger and larger part of ntestinal blood flow was distributed to highly ascularized part of the intestine probably located in the submucosa and adjacent parts of the mucosa. At intense vasodilatation (enous outflow 225 ml/min × 00 g) about 60 per ent of total flow passed through these intestinal structures, the blood of which amounted t 800- 1000 ml/min × 100 g. The blood flow of the rest of the sa-submucosa ncreased concomitantly to 150—200 ml/min × 100 g and that of the ularis to 35—40 ml/min × 100 g

In a pre ous paper (Kampp, Lundgren and Sjöstrand 1967) it was demonstrated that the wash-out curve registered by a scintillation detector after an injection of a solution containing Kr^{85} into the superior mesenteric artery could be resolved into four components. Four independent methods (the registration of the elimination of β-activity local injections autoradiography weight comparisons) were used in an attempt to localize the different components. The following localization was made probable. The first component reflected predominantly a countercurrent exchange of krypton between the scending and descending limbs of the ascular loops in the mucosa at low and moderate flow le ls During asodilatation it mainly mirrored the wash-out of the tracer from a well-perfused part in the submucosa and in adjacent parts of the mucosa. The second and third components mainly reflected the disappearance of Kr^{85} from the mucosa and the muscularis, respectively The slowest components (IV) probably mirrored an elimination of Kr^{85} from the perivascular fat of the mesentery and an absorption of the tracer from the intestinal lumen.

If it can be assumed that the diffusion equilibrium for the tracer between blood and tissue is virtually instantaneous, blood flow can be calculated from the k-value of the slope of the wash-out curve (Kety 1951 see below) In the present paper results will be presented which seem to validate this assumption for Kr^{85} as far as the intestine is concerned. In addition, flow rates and blood flow distribution within the intestinal wall will be discussed in relation to the magnitude of the directly measured total intestinal blood flow

Methods

A. Theoretical considerations

The elimination of an inert gas from constantly and uniformly perfused tissue can be described by the equation.

$$C_t = C_0 \times {}^{kt} \tag{1}$$

(C_t and C_0 = tissue concentration of the gas at time t and 0, respectively, k = clearance constant) provided that the arterial concentration of the gas is zero or negligibly low during the wash-out period (Kety 1951) If the gas leaves the tissue in the blood stream exclusively and if it can be assumed that the equilibrium of the gas bet een blood and tissue is extremely rapid, i.e. negligible concentration gradients of the substance exist in the tissue blood flow (f) in ml/min × 100 g tissue can be calculated from the formula

$$f = \frac{\ln 2 \quad \times 100}{t\,1/2} \tag{2}$$

(= tissue-blood partition coefficient of the gas divided by the specific weight of the tissue t 1/2 = half time of decay expressed in min) t 1/2 is calculated from the straight line obtained hen plotting semilogarithmically the tissue-concentration curv versus time. The olume flow of each of the different components of multiexponential elimination curv can be calculated in similar fashion.

I the case of simultaneous registration of several elimination rates (multiexponential curves) the relativ distribution of flow to the different compartments is estimated in the following w y The amount of tracer present in each compartment at the end of the injection is determined by extrapolating each component to time zero. By relating this amount to the total amount of the tracer the relativ flow of blood to each compartment is calculated.

The theoretical considerations were more fully reviewed in previous paper (Kampp, Lundgren and Sjostrand 1967) For detailed discussions the reader is referred to the original works by Kety (1951 1960)

B. Operativ proc dur

For detailed description of the operativ procedures and the isotope technique (section C) the reader is referred to the work by Kampp, Lundgren and Sjostrand (1967)

Obser tions were carried out on 28 cats anesthetized ith chloralose (40—70 mg/kg) The cats had been deprived of food for at least 24 hrs and had no obvious signs of intestinal infection The experiments were performed on intestinal segments eighing 20—50 g (mainly jejunum) prepared free from adjacent intestinal tissue The mesenteric ein, which in such preparation drains all the blood from the intestinal segment and its lymph nodes, was cannulated and connected to drop-recorded unit operating an ordinate writer From the drop-recorder the blood flowed through short rubber tube into funnel in the j gular ein. Mean arterial blood pressure was recorded from the left femoral artery by means of mercury manometer A small branch of one of the mesenteric arteries supplying the intestine was cannulated with thin polyethylene catheter (PE 10) to permit lose intra-arterial injections of isotopes. Intravenous infusions to induce intestinal vasodilatation were given ia atheter in the left femoral ein Close intra-arterial infusions were made through atheter inserted in retrograde fashion into the superior mesenteric artery The influence of the autonomic nervous system was eliminated by giving atropine (1 mg/kg) cutting the splanchnic nerves bilaterally and by denervating one adrenal gland and excluding the other from the blood circulation by ligatures

The intestinal segment was placed on specially designed wooden plate outside the abdomen in order to secure constant geometrical conditions during the radioactiv measurements. The temperature of the intestine was continuously controlled with thermocouple thermometer (Electrolab Copenhagen, type TE3) placed in the lumen of the gu By means

of heating lamps the temperature was kept t 38 C. Most of the serosal surface of the intestine and its mesentery was covered by Mylar® (12 µm thick Du Pont) to hinder diffusion of the radioacth gas f om tissue to al Parts not ov red by Mylar were protected by gauze soaked with bodywarm saline.

C Isotope t chnique

0.4—0.9 ml of a 0.9 per ce t saline sol tion ontaining Kr^{85} (0.8 mC/ml purchased from the Radiochemical Center Amersham, England) was given as a single injection, lasting 1—2 sec, int the thin polyethylene cathet r (see section B) The γ-emission of Kr^{85} (0.6 per cent of the total adioactivity of Kr^{85}) was recorded by an external scintillation detector th thalliumactivated NaI-crystal (size 1 3/4 × 2) placed 4—6 cm from the intestine th the front surfac of the ystal pa allel to the wooden support of the intestine I order to ensure that only radioactivity from the intestine and ts mesentery was registered by the scintillation detector the whole abdomen, except the intestine was overed by lead plate 5 mm thick Further lead plate 3 or 10 mm thick was placed bet ee the thorax and the detector. Contamination of the air w th Kr^{85} in the room was reduced to a minimum by continuously suctioning the al around the funnel in the jugular ein and the animal's expired air into hood.

The scintillation detector was coupled t pectrometer (Packard, Auto-Gamma Series 410 A) and linear ratemeter (Packard, Model 385) operating one hannel of three channel ink-writer (Rika Denki, Kogyo, Model 34 B) Pulses corresponding t γ-energies above 70 Kev were ounted. In the initial phase of an experimental run, when the hange of radioactivity was rapid, the time constant of the ratemeter was set t 0.3 sec and the paper speed of the recorder to 20 cm/min. These parameters were then changed i a stepwise fashion to 30 sec and 1 cm/min when the tail of the wash-out curve was reached.

D Th tissue-blood partitio oeffici t

In order to be able to estimat blood flow from the wash-out curves of Kr^{85} the tissue-blood partition coefficient of the trace must be know (see equation 2) A far as Kr^{85} is concerned the tissue-blood partition coefficient has been determined in sev al organs. In all tissues except f t a value in the icinity of one has been obtained (see Table I) For that reason an assumed tissue-blood partition coefficient (divided by the specific weight of the tissue) of 1.00 t a hematocrit of 50 pe cent was used for the compartments located in the intestinal wall (I—III) Preliminary periments, in which this partition coefficient was determined, seem to alidate this assumption Furthermore, it can be calculated from the data reported by Yeh d Peterson (1965) on the solubility of Kr^{85} that the tissue-blood parti on coefficient for the tine is around 1 0 Since the fourth component was thought maint to reflect blood flow the perivascular f t an assumed tissue-blood partition coefficient (divided by the specific ight of the tissue) of 5 0 t hematocrit of 50 per cent was used for that compartment (cf Lassen 1967)

It is w ll know that the tissue-blood partition coefficient of Kr^{85} and other lipid soluble substances is dependent on the hematocrit alue of the blood (Kety 1951) Since it as found that hematocrit aried greatl among the cats used in the present study (range of ariation

TABLE I The tissue-blood partition oefficient (divided by the specific weight of the tissue) of Kr^{85} for various organs

Tissue	Kr^{85} tissue blood partition oeff	Hematocrit %	Reference
Brain cortex	0.92	50	Ingvar and Lassen (1962)
white matter	1.26	50	Ingvar and Lassen (1962)
Kidney cortex	0.96	50	Bell and Harper (1965)
Liver	1 04	—	Hollenberg and Dougherty (1966)
Retina	1 0	—	Friedman *et al.* (1965)
Skeletal muscle	1.0	—	Lassen (1967)
Testis	0.85	—	Setchell *et al* (1966)
Fat tissue	5.0	—	Lassen (1967)

18—46) the differences in hematocrit were corrected for utilizing the formula presented by Lassen and Munck (1955). The hematocrit of the blood of the mesenteric vein was accordingly repeatedly determined in all experiments, except in a few early ones in which a value of 31 per cent was assumed, representing an average of all determinations made. Heparinized capillary tubes were filled with blood, sealed in one end by flame and centrifugated at 5000 rpm for fi min. No correction was made for trapped plasma.

E. Calculations

The blood flow corresponding to the different curve components were determined according to equation (2) (Methods, section A) assuming a tissue-blood partition coefficient of 1.00 for components I—III and 5.0 for component IV at a venous hematocrit of 30 per cent (Methods, section D). The relative distribution of blood flow corresponding to the four components, was estimated from the per cent of total activity initially distributed to the different components (Methods, section A).

The total blood flow of the intestinal segment and its mesentery expressed in ml/min × 100 g (F) can be calculated from the data on blood flow (f ml/min × 100 g) and distribution (A per cent) of the four components (I—IV) using the mathematically derived formula

$$F = \frac{100}{\frac{AI}{fI} + \frac{AII}{fII} + \frac{AIII}{fIII} + \frac{AIV}{fIV}} \tag{3}$$

Such a calculation was performed on each experimental run where the directly recorded blood flow exceeded 60 ml/min × 100 g. Below this level of flow the first component did not reflect blood flow but in all probability predominantly countercurrent exchange of Kr^{85} in the intestinal mucosa. The calculated blood flow value was compared with the recorded venous outflow (Results, section A).

The relative weights of the four compartments were calculated in the following way. When venous outflow amounted to a value less than 60 ml/min × 100 g the weight percentage was calculated from the deduced data on the amount of tracer initially distributed to and the blood flow of the different compartments and from the recorded venous outflow. Below 60 ml/min × 100 g the γ-curve did not exclusively reflect vascular events but also in all probability countercurrent exchange in the mucosa, as mentioned above. When the directly recorded total blood flow exceeded 60 ml/min × 100 g the calculations were made using the blood flow value calculated from the γ-curve.

Results and comments

A. Calculated blood flow versus directly recorded total intestinal blood flow

Result. The blood flow calculated from the desaturation curve of intra-arterially injected Kr^{85} was compared with the simultaneously registered venous outflow in all experiments where outflow exceeded 60 ml/min × 100 g. As is evident from Fig. 1 most observations fell within ± 20 per cent of identity (indicated by the upper and lower straight lines). The difference between the recorded and calculated blood flow was not statistically significant (p>0.6). A good correlation existed between the two ways of determining flow (r = 0.94, p<0.001). At blood flows below 60 ml/min × 100 g countercurrent exchange of Kr^{85} in the villi was predominantly reflected in the first component of the wash-out curve (cf. Fig. 3 of this paper and Fig. 7 in Kampp, Lundgren and Sjöstrand 1967). Hence, at these flow levels the elimination curve did not exclusively reflect vascular events and any comparison between calculated and directly recorded flow was therefore not performed.

Comments. It may be argued that the recorded venous outflow was not truly representative of the blood flow of the intestine and its mesentery since the venous blood from the lymph nodes around the mesenteric root was also drained in the

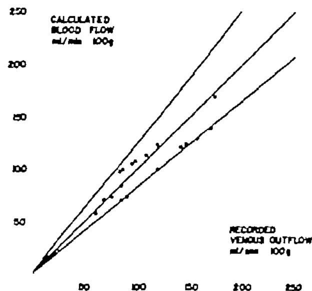


Fig. 1. Cumulated data on the correlation between recorded total intestinal blood flow (abscissa) and blood flow calculated from the wash-out of Kr^{85} (ordinate). For reasons given in the text only experiments in which venous outflow exceeded 60 ml/min × 100 g were included in the figure. Straight lines indicate identity ± 20 per cent.

mesenteric vein and, hence measured by the drop-recorder unit. The weight of the lymph glands amounted however in most experiments to only 10—20 per cent of the total weight of the preparation. Furthermore the "resting" and maximum blood flow values of the lymph nodes reported by Lundgren and Wallentin (1964) (20—60 and 180 ml min × 100 g, respectively) are as great as those of the intestinal preparation.

Theoretically the injection of the tracer should be instantaneous. The error introduced by an injection lasting one to two sec will increase with the augmentation of flow since a larger proportion of total radioactivity will leave the tissue during the injection at high outflow rates. One may therefore *a priori* suspect that the calculated blood flow will clearly underestimate flows above a certain level. This may be a factor of importance in explaining the slight underestimation of recorded blood flow observed in Fig. 1 when venous outflow exceeded 140—150 ml/min × 100 g.

In conclusion, it may be stated that the results, presented in Fig. 1 indicate a rather good agreement betwen the two methods of recording total intestinal blood flow. The results thus seem to validate the assumption of an almost instantaneous diffusion equilibrium for Kr^{85} betwen tissue and blood in the intestine of the cat.

B. Blood flow and flow distribution in the small intestine at various venous outflows

1. First component

Results. The upper panel of Fig. 2 illustrate the relationship betwen recorded venous outflow and the blood flow of the first component as estimated from the slope of the elimination curve of Kr^{85} i.e. assuming that the first component reflected blood flow exclusively. Various degrees of vasodilatation were induced by constant intravenous or intra-arterial infusions of isopropylnoradrenaline. The

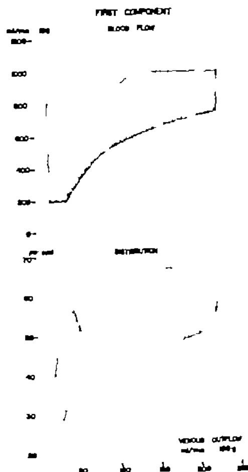


Fig. 2. Cumulated data on the correlation between recorded total intestinal blood flow on the one hand, and blood flow (upper panel, = 67) and distribution of flow (lower panel, = 62) as deduced from the first component, on the other. Flow and distribution were calculated as though the first component reflected intravascular events exclusively (see text). Dots denote means of classed values, shaded areas indicate approximate dispersion of data. Vasodilatations were induced by constant, intra-arterial infusions of isopropylnoradrenaline.

upper panel is based on 67 measurements, classed according to levels of blood flow. Mean values are denoted by dots and the shaded area indicates the approximate dispersion of data.

The per cent of initial activity distributed to the first compartment is in a similar manner plotted *versus* venous outflow in the lower panel of Fig. 2. This diagram is based on 62 measurements. The distribution data of five experimental runs were discarded since the time of injection exceeded three sec.

Two different physiological mechanisms were reflected in the first component (Kampp, Lundgren and Sjöstrand 1967), i.e. a countercurrent exchange of the tracer in the mucosa and blood flow through a well-perfused region localized to the submucosa and the mucosa. In an attempt to roughly estimate the separate contribution of these two mechanisms to the first component at various blood flows, the following calculations were performed. The weight of the third compartment, essentially corresponding to the muscularis, was calculated as described in Methods.

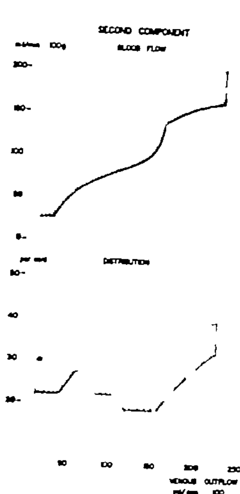


Fig. 4 Cumulated data on the or lation between total intestinal blood flow on the one hand, and blood flow upper panel) and distribution of flow (lower panel) as de duced from the second omponent on the other Dots denote means of lassed alues, shaded areas indicate pproximate disper sion of data. Vasodilatations w re induced by constant, intra-arterial infusions of iso-propylnoradrenaline

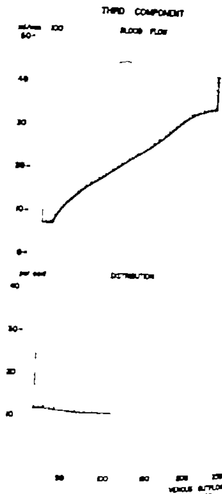


Fig 5 Cumulated data on the correlation bet een total intestinal blood flow on the one hand, and blood flow (pper panel) and distribution of flow (lower panel) as de duced from the third component, on the other Dots denote means of classed alues, shaded areas indicate pproximate dispersion of data. Vasodilatations ere induced by constant, intra-arterial infusions of isopropyl-noradrenaline

to the third compartment, decreased im ltaneously from a alue around 20—30 per cent to about 10 per cent of total a tiv ty

The calculated a erage *weight* of this compartment did not appreciably change with blood flow but remained constantly at 50—55 per cent of the total intestinal weight, exceeding the directly measured relati weight of the muscularis by about 20 per cent (see also Kampp, Lundgren and Sjostrand 1967)



Fig. 6. Cumulated data on the correlation between total intestinal blood flow on the one hand, and blood flow (upper panel) and distribution of flow (lower panel) as deduced from the fourth component, on the other. Flow and distribution were calculated as though the fourth component exclusively reflected blood flow of the perivascular fat of the mesentery (see text). Dots denote means of classed values, shaded areas indicate approximate dispersion of data. Vasodilatations were induced by constant, intra-arterial infusions of isopropylnoradrenaline.

C mments. In a previous paper (Kampp, Lundgren and Sjöstrand 1967) ev dence was presented for the iew that the third component of the v-curv mainly reflected the blood flow of the muscularis. This conclusion is further strengthened when comparing the blood flow of this compartment with that of other smooth muscle tissues, g the uterus. Blood flow alues in the same range of magnitude as that of the third compartment ha been reported for the uterus in several species (Reynolds 1963 Munck t al. 1964 Lysgaard and Lefèvre 1965) Furthermore the blood flow of the muscularis of the cat bladder corresponds to the resting range of the intestinal third compartment (Folkow Lisander nd Lundgren, n-published observ tions)

4 F th mponent

R ult The data on blood flow and distribution as deduced from the fourth component is plotted vers the enous outflow in Fig 6 The blood flow was calculated assuming that this component reflected blood flow w thin the perivascular fat ex lusively e u g tissue-blood partition coefficient of 5 0 at a hematocrit of 40 per cent (see Methods, section D) It can be seen that blood flow increased from resting lue of about 5—10 ml min × 100 g to around 10—15 ml mi × 100 g

during vasodilatation. Concurrently the relative distribution decreased from 5—10 per cent to less than 1 per cent.

The average calculated weight of this compartment amounted to 1?—19 per cent at venous outflows below 100 ml min × 100 g, but decreased to about 9 per cent when venous outflow exceeded 100 ml min × 100 g (see also Kampp Lundgren and Sjöstrand 1967).

Comments The blood flow values of the fourth component concur with those reported for adipose tissue in dog (*Rosell 1966*) and man (Larsen, Lassen and Quaade 1966) and with the values given by Thorburn *et al.* (1963) on blood flow of the perirenal fat provided that one recalculates their data using a partition coefficient of 5 instead of 9. Observations were reported in a previous paper (Kampp Lundgren and Sjöstrand 1967) however which seemed to substantiate the view that an absorption of Kr^{85} from the intestinal lumen was also reflected in this component.

C. Calculated blood flow and distribution of the mucosa-submucosa and the muscularis of the small intestine

Results In those experimental runs in which the weight of the muscularis was directly measured (n = 31) the following calculations were performed. Assuming that the blood flow deduced from the third component of the composite γ-curve reflected muscularis blood flow it was possible to calculate the amount of blood flow distributed to the muscularis by multiplying calculated flow rate by the measured weight of the muscularis. Since total venous outflow was known, the amount of blood flow distributed to the mucosa-submucosa as well as an average blood flow rate of this part of the intestine could then be calculated. No correction for the minor fraction of blood flow distributed to the fourth compartment was made in these calculations.

Fig. 7 summarizes the results. In the upper panel the mean blood flow of the mucosa-submucosa and the blood flow of the muscularis (i.e. third component of the γ-curve) are plotted versus the magnitude of total venous outflow from the intestine. The relative distribution of blood flow to the two portions of the intestine is illustrated in a similar way in the lower panel. It is evident from this figure that the average blood flow of the mucosa-submucosa increased from about 30 ml min × 100 g to approximately 350 ml min × 100 g as total intestinal blood flow increased from 20 to 200—250 ml min × 100 g. The fraction of blood flow distributed to the muscularis decreased simultaneously from about 25 per cent to less than 10 per cent.

Comments The average blood flow of the mucosal submucosal part of the intestinal wall, as deduced from the difference between total flow and muscularis flow was of the same order of magnitude as the blood flow of the second compartment when venous outflow was below 100 ml min × 100 g (compare upper panels of Fig. 4 and 7). This observation suggests that the second compartment ?? reflected the blood flow through the major part of the mucosa-submucosa ??

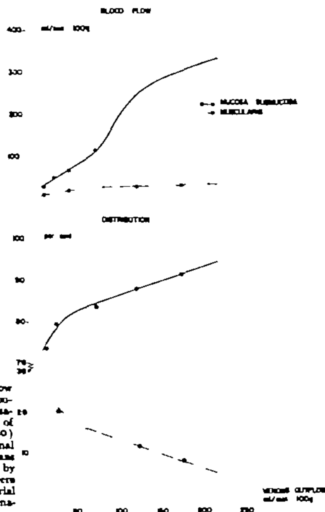


Fig. 7 Calculated blood flow (upper panel) and flow distribution (lower panel) of the mucosa-submucosa (●————) and of the muscularis (○— — — —○) at various levels of total intestinal blood flow Dots indicate means of lumped values. Lines drawn by inspection. Vasodilatations were induced by constant, intra-arterial infusions of isopropylnoradrenaline.

level of venous outflow As total intestinal blood flow exceeded 100 ml min × 100 g mean blood flow of the mucosa-submucosa increased comparatively more than that of the muscularis, probably mainly reflecting the vast augmentation of the blood flow distributed to the earlier discussed, particularly well perfused section in the mucosa and submucosa (see Results, section B of this paper and also Ka pp Lundgren and Sjostrand 1967)

General discussion

In all studies hitherto performed to elucidate the rat of blood flow and the flow distribution within the intestinal wall, lipid insoluble substances or radioactively labelled glass spheres ha been utilized. Grim and co-workers injected DHO i tra arterially (Rayner McLean and Grim 1960 Weiner 1961 Wei er and Grim 1966

or glass beads labelled with Na^{24} (Grim and Lindseth 1958) and determined the amount of the tracer in the different layers of the intestinal wall. Rb^{86} injected intravenously was used in a similar way by Cserma, Wolf and Varro (1965)

In order to be able to calculate flow from the distribution of lipid insoluble substances in the intestinal wall, it must be assumed that the transcapillary transfer of the solute is flow limited and not diffusion-limited. Some studies made on skeletal muscle and intestine indicate however that the transfer of lipid insoluble solutes between tissue and blood is diffusion-limited at high rates of blood flow (cf Renkin 1959, Lassen 1964 Dresel, Folkow and Wallentin 1966 Pitts 1967) probably because of too short a transit time through the exchange vessels. Consequently the extraction of lipid insoluble substances from the blood will be more complete the slower the linear rate of flow Furthermore, it was recently demonstrated that the rate of transfer of lipid insoluble tracers from tissue to blood is affected by other factors than blood flow i.e. the magnitude of net transcapillary movement of fluid (Lundgren and Mellander 1967

The dependency on the linear rate of flow when lipid insoluble substances are extracted from blood, may be a factor that explains the discrepancies between the results of the studies mentioned above and those of the present study Grim and coworkers report blood flow values for the muscularis of the dog between 50 and 0 ml min × 100 g values which, in fact, exceed the average blood flow of the mucosa and submucosa, as recorded by the same technique (cf Fig 7) Moreover a very large proportion (about 40 per cent) of total intestinal blood flow should, ording to the DHO-technique be distributed to the muscularis (cf Fig.)
e results on rats described by Cserma, Wolf and Varro (1965 using Rb^{86} are essentially the same although the flow of the rat intestine is considerably larger than that of the cat. These data may predominantly reflect the fact that the linear rate of blood flow within the muscularis is slower than that of the mucosa-submucosa.

The existence of countercurrent exchange also complicates the use of intra-arterially injected tracers in the study of intestinal blood flow This is obvious from the interpretation of the first component in the Kr^{85} clearance curves, as analyzed in the present study Although most of the results, which strongly suggest a countercurrent mechanism i the mucosa, ha been obtained in experiments with lipid soluble substances antipyrine krypton and oxygen Kampp and Lundgren 1966, Kampp, Lundgren and Nilsson 1967 there are some observations which seem to indicate that a countercurrent exchange in the illi of lipid insoluble tracers may also occur but to a smaller extent (Kampp, Lundgren and Sjöstrand, to be published) Further the dela ed bsorption of DHO from the intestinal lumen, reported by Grim, Lee and Visscher (1955 might indicate that this substance is affected by the countercurrent exchange The possible existence of a countercurrent exchange of DHO which would then also constitute a relati e hindrance for this tracer to reach the tips of the illi, would explain the comparatively low figures for mucosal blood flow (around 40 ml min × 100 g t a enous outflow of approximately 60 ml min × 100 g) reported by Grim and coworkers.

The technique utilizing labelled microspheres (Grim and Lindseth 1958) is not liable to the theoretical drawbacks discussed above. As pointed out by Folkow (1967) it may however be difficult to interpret results obtained by this technique, if a substantial number of wide-bore capillaries and/or very narrow true shunts are present. Nevertheless, some of the results of Grim and Lindseth concur fairly well with those obtained with the present technique, although the two reports may not be strictly comparable, since it is not clear whether the preparation of Grim and Lindseth was denervated; moreover they used dogs. For example the mean flow of the mucosa-submucosa (capillary + A—V bridge flow around 65 ml/min × 100 g at a venous outflow of 57 ml/min × 100 g) given by these authors for fasted jejunum agrees fairly well with those deduced from the results of the present study (Fig 7). On the other hand, their reported flow values for the muscularis (40—50 ml/min × 100 g) are considerably larger than those of the present study.

The results of the present investigation suggest that the range of blood flow of the intestinal muscularis is of approximately the same order of magnitude as that of skeletal muscle, i.e. between 10 and 40 ml/min × 100 g. The flow of the mucosa (as reflected by the second component) on the other hand, is 3—5 times larger than that of the muscularis. The blood flow of the second compartment seems to be of particular importance, since it probably reflects the magnitude of the blood flow of the villi which constitutes the volume flow of blood available for the intestinal absorption. In the resting cat intestine this flow amounts to about 40 ml/min × 100 g and it can be increased at least fourfold by infusion of isopropylnoradrenaline.

During vasodilatation an increasingly large proportion of total intestinal blood flow seems to be distributed to a well-perfused part of the submucosa and the mucosa, possibly corresponding at least in part, to the "Venenbällchen" described by Spanner (1932). The functional significance of this observation is not yet established. It was suggested by Folkow *et al.* (1964) that the intestinal blood flow may be to a large extent *redistributed to such submucosal vascular structures upon* stimulation of the regional sympathetic vasoconstrictor fibres. This redistribution of flow may be of importance in maintaining the amount of oxygen supplied to the liver also when the mucosal blood supply becomes reduced by vasoconstrictor fibre activation (Cobbold *et al.* 1964, Wallentin 1967). However, since the heavily vascularized section of the intestinal wall in part seemed to be located also in the tissue between the crypts (Kampp, Lundgren and Sjöstrand 1967), the first curve component may also reflect blood flow in such portions of the intestinal wall which may be of particular importance during secretory activity, a process known to call for a large volume flow of blood.

This work was supported by grants from the Swedish Medical Research Council (B67-14X-16-03B), from Air Force School of Aerospace Medicine through the European Office of Aerospace Research (OAR), United States Air Force under Contract AF 61(052)-732, from U.S. Public Health Service (HE-05675-06) and from the Faculty of Medicine, University of Göteborg.

References

BELL, G. and A. M. HARPER, Measurement of local blood flow in the renal cortex from the clearance of krypton[85]. J urg Re 1965 5 382—386.

COBBOLD, A., B. FOLKOW O. LUNDGREN and I. WALLENTIN Blood flow capillary filtration coefficients and regional blood olume responses in the intestine of the cat during stimulation of the hypothalamic defence area. Act phy iol. scand 1964 61 467—475

CSERNAY L., F WOLF and V V ..., Der Kreislaufgradient im Dünndarm. Z. Gastroenterologie 1965 3 261—265.

D ELL, P B. FOLKOW and I WALLENTIN Rubidium[86] clearance during neurogenic redistribution of intestinal blood flow Act physiol. cand 1966 67 173—181

FOLKOW B., Regional adjustments of intestinal blood flow Gast oent ology 1967 52 423—432.

FOLKOW B D H LE H, O LUNDGREN S. MELLANDER and I. WALLENTIN, The effect of the sympathetic asoconstrictor fibres on the distribution of capillary blood flow in the intestine A t physiol and 1964 61 458—466.

FRIEDMAN E., H H. KOPALD and T R. SMITH Retinal and choroidal blood flow determined with krypton[85] in anesthetized animals. Inv st Ophthal. 1964 3 539—546.

GRIM, E., J S LEE and M B VISSCHER, W ter exchange between intestinal contents, tissues and blood Am J Physiol 1955 182 359—365.

GRIM, E. and E O LINDSETH Distribution of blood flow to the tissues of the small intestine of the dog. Minn. M d. 1958. 30 138—145

HOLLENBERG, M. and J DOUGHERTY Liver blood flow measured by portal enous and hepatic rterial routes with Kr[85] Am J Physiol. 1966 210 926—932.

INGVAR, D H and N A. LASSEN Regional blood flow of the cerebral cortex determined by krypton[85] A t physiol sc nd 1962 54 325—338.

KAMPP M and O LUNDGREN E idence for countercurrent exchange in intestinal illi Act physiol scand 1966 68 Suppl. 77 103

KAMPP M O LUNDGREN and N J NILSSON Extra scular short-circuiting of oxygen indicating countercurrent exchange in the intestinal illi of the cat. Experie tia (Basel) 1967 23 197—198

KAMPP M O LUNDGREN and J SJÖSTRAND, On the components of the Kr[85] wash-out curves from the small intestine of the cat A t physiol. scand 1968 72 57—281

…TY S S., The theory and pplications of the exchange of inert gas at the lungs and tissues Ph m R 1951 3 1—41

… S S Theory of blood-tissue exchange and its pplication to measurement of blood flow M th med Re 1960 8 223—227

LASSEN N A Muscle blood flow in normal man and in patients with intermittent claudication evaluated by simultaneous Xe[133] and N learances J lin. I est 1964 43 1805—1812

LASSEN N A Studies of peripheral irculation In Belcher E H. and H. Vetter. Radioisotopes in Medical Diagnosis 1967 I press

LASSEN N A and O MUNCK, The cerebral blood flow in man determined by the use of radioacti krypton[85] Act physiol. scand 1955 33 30—49

LARSEN O. A N A. LASSEN and F QUAADE, Blood flow through human adipose tissue determined with radioact xenon Act physiol sc d 1966. 66. 337—345.

LUNDGREN O and S MELLANDER, A gmentation of tissue-blood transfer of solutes by transcapillary filtration and absorption Acta physiol and. 1967 70 26—41

LUNDGREN O and I WALLENTIN Local chemical and nervous control of consecuti... ascular sections in the mesenteric lymph nodes of the cat A giologica (Basel) 1964 1 284—296

LYSGAARD, H and H LEFF ..., Myometrical blood flow in pregnancy measured with xenon[133] Acta obstet gynec. scand. 1965 44 401—407

MENCK, O G PONTONNIER, H L ...GAARD H Lark ... and N A. LASSEN, Détermination du débit du muscle utérin par injection locale de xénon[133] Rev F anç Étud. clin. biol. 1964 9 750—753

PITTS, R. F Discussion of "The indicator fractionation technique for the study of regional blood flow Gastroent rology 1967 52 371 —373

RAYNER, R., L. MACLEAN and E GRIM, Intestinal tissue blood flow in shock due to endotoxin Circulat. Res. 1960. 8 1212—1217

RENKIN, E. M., Transport of potassium[42] from blood t tissue in isolated mammalian skeletal muscle. Ame J Physiol. 1959. 197 1205—1215

REYNOLDS, S. R. M., Maternal blood flow in the uterus and placenta. In Handbook of Physiology 1963. Section 2 (Circulation) Vol. II 1585—1618.

Rosell, S. Release of free fatty acids from subcutaneous adipose tissue in dogs following sympathetic nerve stimulation. *Acta physiol. scand.* 1966. *67* 343—351

Setchell, B. P. G. M. H. Waites and G. D. Thorburn Blood flow in the testis of the concious ram measured with krypton^{85}. Effects of heat, catecholamines and acetylcholine. *Circulat. Res.* 1966. *18* 755—765.

Spanner, R., Neue Befunde über die Blutwege der Darmwand und ihre funktionelle Bedeutung. *Morph. Jb.* 1932. *69* 394—434.

Thorburn, G. D. H. H. Kopald, J. A. Herd, M. Hollenberg, C. C. C. O'Morchoe and A. C. Barger Intrarenal distribution of nutrient blood flow determined with krypton^{85} in the unanesthetized dog. *Circulat. Res.* 1963. *13* 290—307

Wallentin, I., Studies on intestinal circulation. *Acta physiol. scand.* 1967 *69* Suppl. 279 1—38.

Weiner, D., Kinetics of distribution of D_2O in the tissues of the canine ileum (Thesis) Minneapolis: Univ. of Minnesota. 1961. Quoted from Grim, E. The flow of blood in the mesenteric vessels. In Handbook of Physiology 1963 Section (Circulation) Vol. II. 1439—1456.

Weiner, D. E. and E. Grim, Kinetics of distribution of D_2O in canine intestinal tissues. *Amer. J. Physiol.* 1966. *211* 600—606.

Yeh, S. and R. E. Peterson, Solubility of krypton and xenon in blood, protein solutions, and tissue homogenates. *J. appl. Physiol.* 1965 *20* 1041—1047

Acta physiol. scand. 1968. 72. 298—309

From the Institute for Experimental Medical Research, Ullevål Hospital, University of Oslo, Norway

Aortic Baroreceptor Activity in Normal and Hypertensive Rabbits

By

HARALD AARS

Received 23 June 1967

Abstract

AARS, H. *Aortic baroreceptor activity in normal and hypertensive rabbits.* Acta physiol. scand. 1968. 72. 298—309

The baroreceptor activity in the entire left aortic nerve was recorded in 9 normal and 5 hypertensive rabbits. A technique based on rectification and integration of the nerve potentials allowed separate analyses of the mean, systolic and diastolic activities. The nervous activities measured at different diastolic blood pressures showed only small variations during recordings over several hours, and small inter-individual variations. In hypertensive rabbits, the receptors required higher pressure to start firing than in normal rabbits. The activity was lower at all pressures in hypertensive animals. The difference from normal rabbits increased with increasing pressure and was most pronounced for the systolic part of the firing. Mechanisms for causing resetting of baroreceptor activity in hypertension are discussed. It is concluded that ... ination of hypertension ... wall changes and exhaustion or destruction of some receptors is ... abl... responsible for the resetting.

The baroreceptors of the carotid sinus and aortic arch are stretch receptors, responding to increases of arterial pressure with increased firing. At low pressures the activity is small but as the pressure is increased more and more receptor units are recruited and the firing rate of each increased. The activity is pulse-synchronous at low and normal pressures but nearly continuous at high pressures. (For general references see Heymans and Neil 1958.) The firing rate of individual fibres increases almost linearly with mean pressure from 30—40 to 150—200 mm Hg. In multifibre preparations the increase follows an S-shaped curve on account of different thresholds of individual receptors (Franz and Vischer 1962, Spickler and Kezdi 1967).

In dogs with experimental hypertension the receptors start firing at higher pressure than usual, and a pulsatile activity is still present at pressures which in normotensive animals give rise to continuous firing (McCubbin, Green and Page 1956, McCubbin 1958). The receptors evidently take time to change their activity. After 6 hrs of angiotensin-induced hypertension the activity was the same as in

normal dogs at similar pressures, but after 1—3 days of renal hypertension resetting had developed. Resetting here refers to alteration of the relationship between arterial pressure and receptor activity.

It is not known whether resetting affects the activity during systole and diastole to the same degree, neither is it clear whether the resetting is equal at all pressures. In theory resetting could imply a) that the increase of activity resulting from a given increase of pressure was lower at all pressures, i.e. a reduced sensitivity, b) that a higher pressure was required to start the receptors firing, although with further rise of pressure the nervous response to pressure increments was normal, i.e. an increase in threshold, or c) a combination of a) and b). In an attempt to distinguish between these possibilities, quantitative registrations of aortic baroreceptor activity have been undertaken in normotensive and hypertensive animals. Rabbits were used, as their aortic nerves are separate in the neck.

Materials and methods

Recordings from left aortic nerve were obtained in 14 white adult rabbits of the same breed. Five had been made hypertensive; nine served as normal controls. The methods described by Campbell and Santos-Buch (1959) were used for producing hypertension. The rabbits were anesthetized with sodium pentobarbitone (Nembutal) 30 mg/kg body weight. The left kidney was wrapped in silk and soaked in turpentine, and contralateral nephrectomy was performed 3—4 weeks later. Eighteen rabbits were prepared for the experiments. Eight died following operations, another died under anesthesia before nerve recordings could be made, one failed to develop hypertension, and three suffered nerve damage during preparation.

The nerve activity was recorded 5—13 days after nephrectomy. For recordings of the activity of the aortic nerve, the animals were anesthetized with 3 ml 1 % chloralose and 3 ml 25 % urethane per kg. One-half to one-third of the total amount was given i.v., the rest intraperitoneally. When necessary, urethane was supplemented during the experiment. The animals were intubated or tracheotomized, but respiration was not assisted. Rectal temperature was kept between 36.5 and 37.5 C by heating lamp and covering towels. The left aortic nerve was dissected free in the neck. The nerve is easily damaged, and since reduction in the number of active fibres would prohibit quantitative comparisons between animals, it was handled with extreme care. Loose connective tissue and vessels were removed, but the nerve sheath was not opened. The nerve was cut cranially and placed on 0.4 mm thick platinum electrodes 10 mm apart in warm liquid paraffin. The potentials were recorded with differential amplifier, rectified and integrated (Aars and Leraand 1968). The full and rectified neurograms and the integrated activity were recorded on 4-channel jet ink writer (Elema Mingograph) together with right carotid arterial pressure measured with Statham transducer. Paper speed was 50 or 100 mm/sec. As the right aortic nerve is thinner and more difficult to prepare, it was often damaged, and the results are not included in this report.

Since integrated mean activity is proportional to heart rate (Gero and Gero 196) measured mean activity was by calculation normalized mean nervous activity per sec heart rate of 300 beats per min. Systolic activity was measured as the integrated receptor response to the systolic pressure rise, and diastolic activity as the activity in an immediately preceding period. At low pressures, where the systolic activity is small, the recording time must be short in order to avoid inclusion of the subsequent silent period in the systolic activity. At high pressures, however, the activity related to the systolic rise in blood pressure lasts longer and would be underestimated by the short recording period warranted by low activities. 0.04 sec was therefore selected for recording of systolic and diastolic activities (Fig. 1). The nerve activity was measured at various blood pressure levels, obtained by stepwise withdrawal and reinfusion of blood through the right jugular vein. Because of wide variations in receptor response to high pressures obtained with sympathomimetic drugs, recordings at above normal pressures, although desirable, could not be used. After 30 sec for reaching steady state three measurements of each activity were made on the curves from each pressure level.

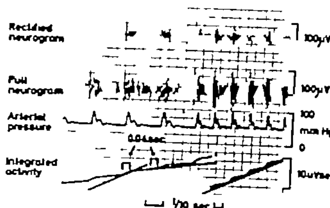


Fig. 1 Recording of left aortic ners activity M an activity per second is found by measuring the slope of the line draw along the curs for integrated activity in the right lower orner of the Figu e. Systolic and diastolic ac tivities are measured at higher paper speed, as the slope of the lines dra n through the first 0.04 sec of systolic increase of activity and the 0.04 sec immediately pre eding systole

To facilitat omparisons between animals, the activities wer grouped in 10 mm Hg steps (40—49 50—59 etc.) and their mean alue calculated. The noise of the recording system was recorded t the end of experiments by cutting the ners distally to the recording level without disrupting tissue ontact. The heart was then removed and cut into three the atria, the left entricle with the septum, and the right entricle. The parts wer rinsed in saline, dried with filter paper and weighed.

Results

Examples of left aortic ners recording in a normotensi e rabbit and in a rabbit made hypertensive are presented in Fig 2. The increase in activity with rise of pressure was steeper and started at lower pressure in the normotensive animal.

Fig. 2. Observations of integrated aortic ners activities from normotensiv rabbit (A) and hypertensive rabbit (B) plotted at the arious diastolic pressures obtained through bleeding (open symbols) and reinfusion (olid symbols) At low pressures the activities ere equal (▼) The lines are drawn to best isual fit.

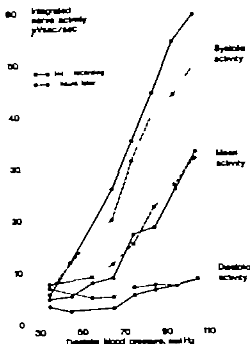


Fig. 3. Mean values of aortic integrated nerve activities from two recordings made three hours apart. The observations are grouped in 10 mm Hg steps. Normotensive rabbit.

Systolic activity increased linearly with pressure in the normal rabbit, but in the other appeared to flatten out at a lower level of activity. The range of mean and diastolic activities was small at each pressure level, but was larger for systolic activity. The noise in the recording (Fig. 2A) was 0.7 μV.sec/sec. It was usually negligible in all the recordings, and was not taken into account. In only one experiment was the noise recording high, and in that instance it was subtracted from the measured activities.

The reproducibility of the recordings was tested by inducing similar pressure variations after an interval of 2—3 hrs. Fig. 3 shows the relationship between diastolic pressure and mean values of integrated activity for two runs, 3 hrs apart. Recordings obtained in several other rabbits also showed only slight and insignificant changes with time.

Similar patterns of activity to those demonstrated in Fig. 2 were observed in the other rabbits in the two groups. The results, at 10 mm Hg pressure intervals, are listed in Table I. Not all animals were represented at each pressure level. In calculating the mean activities for the hypertensive rabbits, therefore, the average activity for two pressure levels was used (40—59, 60—79, etc.), as the changes in activity at each 10 mm Hg increment were small. Four or five hypertensive animals were thus represented at each pressure level.

Phasic and mean nerve activity at various diastolic pressures in normal and hypertensive animals are shown in Fig. 4 and 5. The mean activity of the left aortic nerve

TABLE I Integrated aortic nerve activities in normotensive and hypertensive rabbits

	Diastolic BP mm Hg	Normotensive group: Number of animals	Nerve activity µV sec/sec		Hypertensive group: Number of animals	Nerve activity µV sec/sec	
			mean	S.E.		mean	S.E.
Mean activity	30— 39	8	4 1	0.6			
	40— 49	8	5.3	0.8	4	3.0	0.6
	50— 59	8	7 4	0.6	3	3.7	1.2
	60— 69	7	9 0	0.4	3	5.7*	1.9
	70— 79	8	14 1	1.5	5	6.4	1.3
	80— 89	8	18.2	1 7	3	8.9*	2.7
	90— 99	6	25.2	1.8	4	11.6	2.2
	100—109	3	30.7	5.5	3	14.2	2.7
	110—119				3	16.2	2.9
	120—129				3	18.5	2.4
	130—139				5	20.8	2.5
	140—149				5	23.7	3 6
	150—159				3	27.6	3.7
Systolic activity	30— 39	9	6.8	1.9			
	40— 49	8	14.6	2.9	4	3 8	1.6
	50— 59	7	22 4	1 5	3	8 4	2.8
	60— 69	7	30 5	2.3	3	15.3	3.3
	70— 79	8	38.6	2 7	5	17 7	4.8
	80— 89	8	45 2	3 6	3	*6 0	4.8
	90— 99	6	51 2	4 7	4	26.3	4.5
	100—109	3	51 5	5.2	3	27 4	4.4
	110—119				3	28 0	3.3
	120—129				5	31.5	3.3
	130—139				5	32.4	3.5
	140—149				3	37.0	3 6
	150—159				3	38.1	1.0
Diastolic activity	30— 39	9	2 6	0 5			
	40— 49	8	2 2	0 4	4	2 6	0.3
	50— 59	7	2 0	0 5	3	2.3	0.4
	60— 69	7	3 0	1 0	3	2 2	0.5
	70— 79	8	3 6	0.7	5	2 1	0 2
	80— 89	8	5.0	0 9	3	2.3	0.5
	90— 99	6	6 9	1 1	4	3.2	0.6
	100—109	3	8 9	2 3	3	2.5	0.4
	110—119				3	7 1	2 0
	120—129				3	6.3	1.6
	130—139				5	8 4	2 6
	140—149				5	11.9	3.3
	150—159				3	12.2	4.7

Significant difference between the groups ($P < 0.02$)
Significant difference between the groups ($P < 0.05$)

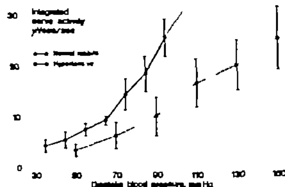


Fig. 4. Integrated mean aortic nerv activity in 9 normotensiv rabbits and 5 hypertensiv rabbits, at 10 and 20 mm Hg intervals respectively. In Fig. 4 and 5, ±2 S.E. are plotted only when the mean is based on observations from more than 3 animals.

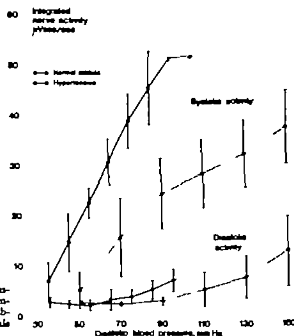


Fig. 5. Integrated systolic and diastolic nerv activities in 9 normotensiv rabbits and 5 hypertensiv rabbits, at 10 and 20 mm Hg intervals respectively.

of normal rabbits rose steadily with increasing pressures, rising most markedly from about 65 mm Hg. Systolic firing tarted t bout 30 mm Hg diastolic pressure and increased almost linearly w th pressures p t about 100 mm Hg. The firing n diastole was low and unchanged to about 70 mm Hg. The activity of the aortic nerv in normal abbits showed only small ariations t identical pressures, as evidenced by low standard errors of the mean (Table I and Fig. 4 and 5).

The systolic and diastolic pressures, when measured immediately prior to the recording of nerv actı ty had increased to n erage of 196/133 mm Hg n the hypertensiv e group (Table II) nd the animals showed left ntricular hype trophy. Left/right entricular weight ratio was 41 higher in the hypert

TABLE II Body weights, arterial pressures and left/right ventricular weight ratios for the normotensive and hypertensive rabbits

Rabbit	Body weight g	Left/right ventricular weight ratio	Arterial blood pressure mm Hg
Normotensive			
411	4000	[illegible]	170/100
413	3600	[illegible]	105/80
434	3200	3.0	145/115
439	3400	3.4	140/110
440	3800	3.2	145/115
441	3000	3.4	140/107
444	3350	3.4	125/95
519	3000	3.2	120/80
522	3620	3.5	120/90
Mean ± S.E.	34[illegible]7 ± 122	3.4 ± 0.05	Systolic 129 ± 4.7 Diastolic 99 ± 4.6
Hypertensive			
437	3200	5.0	205/160
438	3600	5.2	[illegible]/155
514	3280	4.8	[illegible]05/165
517	2600	4.8	190/135
532	4030	4.4	175/1[illegible]0
Mean ± S.E.	3342 ± 224	4.8 ± 0.13	Systolic 196 ± 6.0 Diastolic 153 ± 5.2

[illegible]up, but body weights in both groups were nearly equal (Table II). The heart rates usually varied during recording of nervous activity, and mean difference between highest and lowest frequency was 20.9 (range 0—38) beats per min. Mean heart rate was calculated for each animal, based on the frequency at each recording of nervous activity. Mean heart rate in the groups of normotensive and hypertensive rabbits was 286±24 (S.D.) and 272±26 (S.D.) beats per min respectively. Pulse pressures were highest in the hypertensive group at all comparable pressures.

The receptor activity was low and stable at low pressures, and almost equal for the two groups. The first rise in activity with increasing pressure was observed at about 20 mm Hg higher pressures in the hypertensive than in the normal rabbits, and at all subsequent pressures the activity in the hypertensive rabbits was lower than normal. The slope of the activity pressure curves differed in the two groups (Fig. 4 and 5). There was a significant difference for mean and systolic activities ($P<0.02$, Student's T-test, Table I). The curves for diastolic activities showed a clear tendency to separate (Fig. 5) and at 90 and 100 mm Hg the difference was significant ($P<0.05$).

The reduction of activity in hypertensive animals was found to be independent of variations in interval between nephrectomy and nerve recording (5—13 days).

Discussion

Baroreceptor resetting in hypertension

The aortic nerve activity was clearly different in the two groups. In hypertensive rabbits, a higher pressure than normal was required to start the aortic baroreceptors firing and the receptor activity was lower in the hypertensive group than in normotensive animals at similar pressures. In this respect, the results obtained by McCubbin, Green and Page (1956) and McCubbin (1958) in hypertensive dogs were confirmed. The present investigation showed in addition that the difference in the receptor activity between the groups increased with rise of pressure (Fig. 4 and 5). The greatest part of the resetting was caused by reduction of systolic activity (Fig. 5) both when absolute values were considered and when the relative reduction of systolic and diastolic activities were compared. The response in normal rabbits to pressures above 100 mm Hg was not examined, but it has been shown (Trank and Visscher 1962, Spickler and Kezdi 1967) that the normal aortic nerve activity persists in rising linearly in the higher pressure range which was examined in the hypertensive animals. The difference between normal and hypertensive rabbits would therefore be expected to be even greater at these high pressures (100—150 mm Hg diastolic pressure) than in the range where comparison can be made (Fig. 4 and 5).

Comments on the methods

Quantitative comparisons of aortic nerve activity require homogenous fibre populations in the animals. The aortic nerve of the rabbit contains all the fibres from the aortic arch baroreceptors, and practically no other fibres. Extra neurally recorded, as in the present investigation, the neurogram from the aortic nerve will consist of potentials from myelinated fibres, as the potentials from non-myelinated fibres will barely if at all, exceed the noise level. Little is known about the non-myelinated fibres of the aortic nerve, except that like the myelinated they have a depressor effect on systemic blood pressure when electrically stimulated (Neil, Redwood and Schweitzer 1949, Douglas, Ritchie and Schaumann 1956). The myelinated fibres range in thickness from 2—6μ, and the number from 150—600 (Sarkar 1922, O'Leary, Heinbecker and Bishop 1934). The left nerve is thicker than the right.

Possibly efferent fibres were excluded by always cutting the nerve and recording from its distal part. The small differences in activity from one animal to another suggest that the nerves were not damaged by dissection or recording procedures, and that the number of myelinated fibres in the left aortic nerve is more constant than commonly assumed.

Knowledge of the total nerve activity is necessary for examining the resetting of baroreceptors in hypertension, as the activity of the efferent part of the reflex is determined by the bulk afferent input. Conventional studies of single fibres offer exact information on receptor threshold, firing rate and fibre thickness, but for the present purpose would have necessitated studying a vast number of preparations.

The results of recordings in the entire nerve followed the pattern observed by others in single fibres of the aortic nerve (Bloor 1964 Homma and Suzuki 1966) and carotid sinus nerve (Bronk and Stella 1932 Landgren 1952, Trank and Visscher 1962, Spickler and Kezdi 1967)

In addition to varying numbers of fibres, several other factors, such as difference in activities measured subsequent to bleeding and reinfusion respectively (Fig. 2) height and rate of rise of pulse pressure, and state of the vascular wall and its smooth muscles, contribute to the variations within a group. The variation due to difference in heart rate was accounted for but the other variables were not. They are probably of minor importance however since uniform results were obtained in all animals in a group.

Mechanisms of r sett ng

McCubbin (1958) showed that the resetting in hypertensive dogs persisted when the carotid sinus was perfused with standard, pulsatile pressures. As baroreceptors are very sensitive to pulsatile pressures (Bronk and Stella 1932, Ead, Green and Neil 1952, Trank and Visscher 1962, Spickler and Kezdi 1967) their activity in hypertensive animals would be further reduced by lower pulse pressures and lower rate of pressure rise. However pulse pressures were higher in the hypertensive than in the normotensive rabbits, both at resting pressures (Table II) and during actual nerve recordings. dP dt has been found to follow variations in pulse pressure (own unpublished results) It is therefore concl ded that the changes in pressure pattern would if anything, have counteracted the effect of the resetting of baroreceptor activity in hypertensive animals.

The resetting was partly characterized by the receptors starting to fire t a higher pressure than normal (Fig 4 and 5) Whether this was due to true elevation of receptor threshold, or caused by other factors, could not be evaluated in these experiments. However increased threshold would have resulted in equal slopes of the pressure-acti ity curves for normal and hypertensive rabbits, with the systolic and diastolic parts similarly affected and could not have been the sole cause of the resetting.

Resetting might be d to changes in efferent sympathetic activity In the carotid sinus, which has a separate sympathetic innervation, sympathetic control has been shown to play a minor role (Palme 1944 Floyd and Neil 195– Kezdi 1954 Mazella and Mullin 1956, Moncada and Scher 1963) The effects on the aortic wall of the sympathetic nerve plexus in the aortic arch are not clear If mediated through the aortic nerve, sympathetic activity could not have disturbed the wall in the present study as the nerve was cut. A h moral factor might possibly account for the reduced acti ity in hypertension, since epinephrine anesthetics and several other humoral agents are known to influence the activity of baroreceptors, either directly or via effects on the wall (Heymans and Neil 1958 Anokhin and Shumilina 1962, Trank and Visscher 1962, Biscoe and Millar 1964) Kezdi (1962) however showed that when the carotid *sinus had been protected* from the developing hypertension by

carotid constriction, there was no resetting of the sinus nerve activity He concluded that the high pressure must act directly on the receptor area in order to induce resetting. It is noticeable that when humoral factors are found to influence the firing rate the effect is immediate, whereas in hypertension resetting takes several days to develop.

A reduction in the number of receptors would produce resetting. Hilgenberg (1958) and several Russian authors (cited by Simonson and Brožek 1959) found degenerative histological changes of receptors in aortas from hypertensive patients and animals. If applicable to the hypertensive rabbits, such changes would have resulted in a slope of the activity-pressure curves that was less steep than normal, and would have explained the apparently lower maximum activity in the hypertensive group (Fig. 2, 4 and 5) Sellors (1964) did not find histological abnormalities of nerve endings, and McCubbin (1958) suggested that the loss of receptors might only be functional. From a simultaneous registration of two fibres in the sinus nerve he postulated that low threshold receptors, showing nearly continuous activity at high pressures, might become inactive through fatigue in persistent hypertension. If this had been the case in the present experiments, the systolic reduction of activity would have been caused by the falling out of a continuously firing receptor Systolic and diastolic activity should consequently have been equally reduced. The reduction of activity was more pronounced, however in systole than in diastole (Fig. 5) and mere reduction in the number of receptors could not have offered a complete explanation for the resetting

Because baroreceptors are stretch receptors, reduced distensibility of the hypertensive aortic wall automatically lowers the receptor response to an increase of pressure. In hypertension, most arteries, including the aorta, have increased content of water sodium and possibly other electrolytes, and because of structural changes they are less distensible than normal (Tobian 1960 Karnbaum 1961 Feigl, Peterson and Jones 1963, Greene et al. 1966 Postnov 1966) Peterson (1966) thought that the resetting of carotid sinus baroreceptor activity in hypertensive dogs might be due to the wall being stiffer than normal, similar to his observations of reduced increase of diameter and receptor activity when noradrenaline was applied to the wall of the carotid sinus. Direct comparisons of these parameters in normal and hypertensive animals have not been made. However circular strips of aortic tissue from the hypertensive rabbits were thicker and less distensible than strips from the normal rabbits (own unpublished results) If at the same time more [illegible] the aortic wall would show a decrease in both amplitude and speed of the pulsatile expansions, and the systolic receptor discharge would be lowered more than the diastolic in accordance with the observations illustrated in Fig 3

It is therefore concluded that structural changes of the arterial wall could play an important role in the resetting of aortic baroreceptor activity in hypertensive rabbits.

The work was supported by the Norwegian Council on Cardiovascular Diseases J. L. Tiedemanns Tobaksfabrik, Joh H. Andresens medisinske fond, and the University of Oslo, and was carried out when the author was research fellow of the University

References

AARS, H. and S. LEERAAND, A method for quantitati analysis of aortic nerve activity J. Appl. Physiol. 1968. In press.
ANOKHIN P. K. and A. I. SHUMILINA, Physiological pecularities of aortic arch baroreceptor information on the state of blood pressure. Arch. Int. Pharmacodyn. 1962. 90. 1—7
BISCOE,T. J. and R. A. MILLAR, The effect of Halothane on carotid sinus baroreceptor activity J. Physiol. (Lond.) 1964 173 24—37
BLOOR, C. M. Aortic baroreceptor threshold and sensitivity in rabbits at different ages. J. Physiol. (Lond.) 1964 174 136—171
BRONK, D. W. and G. STELLA, Afferent impulses in the carotid sinus nerve. I. The relation of the discharge from single end organs to arterial blood pressure J. Cell. comp. Physiol. 1932. 1 113—130.
CAMPBELL, W. G. and C.A. S. TON-BUCH, Widely distributed necrotizing arteries induced in rabbits by experimental renal alterations. I. Comparison with the ascular lesions induced by injections of foreign serum. Amer. J. Path. 1959 35 439—466. II. Relationship of the arterial lesions t perirenal inflammation. Amer. J. Path. 1959 35 769—790.
DOUGLAS, W. W. J. M. RITCHIE and W. SCHAUMANN, Depressor reflexes from medullated and non-medullated fibres in the rabbit aortic nerve. J. Physiol. (Lond.) 1956. 132 187—198.
EAD, H. W. J. H. GREEN and E. NEIL, A comparison of the effects of pulsatile and non-pulsatile blood flow through the carotid sinus on the reflexogenic activity of the sinus baroreceptors in the at. J. Physiol. (Lond.) 1952. 118. 509—519.
FEIGL, E. O. L. H. PETERSON and A. W. JONES, Mechanical and chemical properties of arteries in experimental hypertension. J. li I at 1963 42 1640—1647
FLOYD W. F. and E. NEIL, The influence of the sympathetic innervation of the carotid bif rcation on chemoreceptor and baroreceptor activity in the cat. Arch. int. Pharmacodyn. 1952. 91 230—239
GERO J. and M. GEROVA, The role of parameters of pulsating pressure in the stimulation of intracarotid receptors. Arch. int. Pharmacody 1962 140 35—44
GREENE, M. A. R. FRIEDLANDER, A. J. BOLAX, C. G. HADJIKOBOR and O. A. LUSTIG, Distensibility of rteries in human hypertension. Proc. Soc. exp. Biol. (N.Y.) 1966. 121 580—585
HEYMANS, C. and E. NEIL, Reflexogenic as of th ard ovascular system J. & A. Churchill, Ltd., London. 1958.
LOENBERG, F. Neurohistologische Studien über die Pressorezeptoren des Carotissinus bei ypertonikern. Acta neuroveg. (Wien) 1958. 19 1—14
OMMA. S and S. S. AL. Phase properties of aortic and atrial receptors observed from their afferent discharge J. p. J. Physiol 1966 16 31—41
KARNBAUM S. Elastizität und Morphologie des Aortenwindkessels beim Bl thochdruck. Arch Kreisl. F sch 1961 34 18—74
KEZDI, P. Control by the superior cervical ganglion of the state of contraction and pulsatile expansion of the arotid sinus arterial wall Circulat. Res. 1954 2 367—371
KEZDI, P. Mechanism of the arotid sinus in experimental hypertension Circulat. Res. 1962. 11 145—150
LANDGREN S. On the excitation mechanism of the carotid baroreceptors Acta physiol. scand. 1952 26 1 34
MAZZELLA, H. and R. G. M rs. Effects of emoval superior sympathetic ganglion on carotid sinus. Arch. int. Pharmacody 1956 107 54—61
McCUBBIN J. W. J. H. GREE and I. H. PAGE Baroreceptor function in chronic renal hypertension. Circulat. Res. 1956 4 205—210
McCUBBIN J. W. Carotid sinus participation experimental renal hypertension. Circulation 1958. 17 791—797
MONCADA, A. and A. M. SCHER. Reflex on rol of the sensitivity of the carotid pressoreceptors. Circulatio 1963 28 771
NEIL, E., C. R. M. REDWOOD and A. SCHWEITZER. Effects of lectrical stimulation of the aortic nerv on blood pressure od respiration cats and rabbits nder chloralose and nembutal anaesthesia. J. Physiol. (Lond.) 1949 109 392—401
O'LEARY J. P. HEINBECKER and G. H. BISHOP. The fiber constitution of the depressor nerv of the rabbit. Amer. J. Physiol 1934 109 274 285
PALME, F. Zur Funktion der branchiogenen Reflexzonen für Chemo- nd Pressoreception. Z. ges. exp. Med. 1944 113 415—461
PETERSON L. H. Physical factors which influence ascular caliber and blood flow Circulat. Res. 1966. Suppl. I 3—11

POSTNOV, J. W., Das Verhalten einiger Fermente und Elektrolyte der Gefässwand bei der experimentellen Hypertonie der Ratte. *Virchows Arch. path. Anat.* 1966, *341*, 237—244.
SARKAR, B. B., The depressor nerve of the rabbit. *Proc. roy. Soc. B* 1922, *93*, 230—234.
SELLORS, T. H., Hypertension and coarctation of the aorta. *Brit. J. Surg.* 1964, *51*, 726—731.
SIMONSON, E. and J. BROZEK, Russian research on arterial hypertension. *Ann. intern. Med.* 1959, *50*, 129—193.
SPICKLER, J. W. and P. KEZDI, Dynamic response characteristics of carotid sinus baroreceptors. *Amer. J. Physiol.* 1967, *212*, 472—476.
TOBIAN, L., Interrelationship of electrolytes, juxtaglomerular cells and hypertension. *Physiol. Rev.* 1960, *40*, 280—312.
TRANK, J. W. and M. B. VISSCHER, Carotid sinus baroreceptor modifications associated with endotoxin shock. *Amer. J. Physiol.* 1962, *202*, 971—977.

Acta physiol. scand. 1968. 72. 310—321

From the Department of Pharmacology University of Lund, Sweden

The Effect of Calcium on the Skeletal Muscle Membrane after Treatment with Phospholipase C

By

E. X. Albuquerque and S. Thesleff

Received 25 June 1967

Abstract

Albuquerque, E. X. and S. Thesleff *The effect of calcium on the skeletal muscle membrane after treatment with phospholipase C* Acta physiol. scand. 1968. 72. 310—321

The effects of phospholipase C on the muscle membrane were studied by the recording of the resting membrane potential, the input resistance and the action potential generation in single fibres of the isolated extensor digitorum longus and soleus muscles of the rat. Incubation with phospholipase C abolished spike generation, depolarized the fibres by 15—20 mV and increased the ionic permeability of the membrane as shown by a fall of about 40 per cent of the input resistance. Removal of the enzyme by washing the preparation with Krebs-Ringer solution did not restore membrane excitability, the resting membrane potential nor the input resistance of the cells. When the external calcium concentration was raised from the normal 2 mM to 15 mM muscle fibres repolarized and action potentials with reduced rate of rise and amplitude recorded. No recovery of the input resistance occurred. When the calcium concentration brought back to 2 mM the fibres again depolarized and became inexcitable. Potassium (10 or 15 mM), caesium (10 mM) or rubidium (6 mM) antagonized the aforementioned effects of 15 mM calcium. It was concluded that phospholipase C irreversibly affected the muscle membrane and that an increase in the external calcium ion concentration only functionally restored some of the membrane properties, i.e. spike generation and the resting membrane potential. The results support the view that the hydrophilic head of certain membrane phospholipids connected with passive ion transport in the electrogenic membrane.

Studies on the mammalian skeletal muscle membrane have shown that phospholipase C (PhC) effectively blocked the ionic carrier mechanisms responsible for the generation of the action potential (Albuquerque and Thesleff 1967). Since PhC is an enzyme which hydrolyses the ester bond between the glycerol and the phosphate groups of some phospholipids, our findings reinforced the previous suggestions of Tobias (1958, 1964), Tobias, Agin and Pawlowski (1962), Mullins (1961), Goldman (1965), Feinstein (1964) that the hydrophilic head of membrane phospholipids acts as an ion exchange site directly connected with passive ion transport in the electrogenic membrane. Although the effect of PhC on action potential generation in

Research Fellow of IBRO/Unesco. Present address: Dept. of Pharmacology, State University of New York at Buffalo, Buffalo, New York 14214, USA.

many respects was similar to that produced by local anesthetics (Shanes *et al.* 1959 Taylor 1959) the enzyme differed from the former because it reduced the resting potential and increased the ionic permeability of the muscle membrane.

Since calcium ions are known to be involved in the process of membrane excitation as a stabilizing element (Frankenhaeuser and Hodgkin 1957 Shanes 1958 a, b, Tobias *et al.* 1962) and it has been proposed that the site of action of this ion in the membrane is the phospholipid head molecule (Tobias *et al.* 1962, Aceves and Machne 1963 Koketsu, Kitamura and Tanaka 1964 Rojas and Tobias 1965 Shah and Schulman 1965 Albuquerque and Thesleff 1967) it was thought of interest to analyze the effect of this bivalent ion in connection with the action of PhC on the muscle membrane. The results obtained showed that PhC irreversibly affected the muscle membrane but that high concentrations of calcium could partially restore the action potential generation previously abolished by PhC. The univalent ions potassium, caesium and rubidium antagonized this effect of calcium.

Methods

The experiments were made *in vitro* using the extensor digitorum longus and the soleus muscles of young adult rats. The experimental conditions and the electrical recording techniques have been described by Albuquerque and Thesleff (1967).

For measuring the action potential and the input resistance two microelectrodes were inserted into the same fibre with 50—100 μ spacing. One of the electrodes was used for current passing and the other for potential recording.

When the action potential was recorded the resting membrane potential was set to a value of 70—80 mV by passing a constant anodal current through the membrane, the current terminating in a 30 msec cathodal shock adjusted in steps of increasing magnitude until an action potential was produced. The duration of the hyperpolarizing current was in general 100 msec but in some experiments the time was prolonged up to 3 min. The threshold depolarization for the generation of the action potential was taken as the amplitude of the potential step preceding the selfregenerative response. The amplitude of the action potential was measured from its threshold of generation to summit. The rate of rise of the action potential was determined by the use of a differential RC circuit (100pF 100KΩ). The time constant of the recording circuit was about 30 μsec.

The input resistance of single fibres was measured with pulses of varying intensity and 200 msec duration covering at least three anelectrotonic potentials between 2 and 10 mV. The average result of such a series was used. The electric time constant (τm) of the membrane was obtained by measuring the time required for the hyperpolarizing membrane potential change to reach 85 % of its plateau value (Boyd and Martin 1959).

The basic Ringer solution had the following composition in mM: NaCl—135.0 KCl—5.0 $NaHCO_3$—15.0 NaH_2PO_4—1.0 $CaCl_2$—2.0 $MgCl_2$—1.0 and Glucose 11.0. When the ionic content of the basic Ringer solution was varied the solution was kept isosmolar by appropriate changes of NaCl. The temperature of the solutions were maintained at 22 ± 5 °C and they were continuously oxygenated with a gas mixture of 95 % O_2 and 5 % CO_2 which gave them a pH of 7.0—7.3. When the ionic composition of the basic Ringer solution was changed the muscles were allowed to equilibrate for 1 hr in the new medium before any recordings were made.

Phospholipase C from *Clostridium welchii* (phosphatidylcholine cholinephosphohydrolase E.C. 3.1.4.3.) was obtained from Sigma Chemical Company, St Louis, Mo. According to the manufacturer 1 mg of the enzyme liberates approximately 0.5—2.0 μl of water-soluble organic phosphorus from egg yolk lecithin per min at pH 7.3 at 37 °C. The doses of the enzyme added to the bathing solution are expressed in μg/ml of the total material. The time of incubation with PhC was, unless otherwise stated, 60 min and after that period the measurements were made without change of bathing solution. The total time of incubation was thereby increased by no more than 30 min. Tetrodotoxin was obtained from the Sankyo Co. Ltd, Tokyo, Japan.

Results

Action potential

The effect of PhC on the action potential was examined in the isolated extensor digitorum longus and the soleus muscles of the rat. Most of the analysis, using different external ion concentrations, were made only on the extensor digitorum longus.

Since incubation with PhC and several of the experimental procedures depolarized the muscle fibres the membrane potential of individual fibres was locally set to 70—80 mV by passing a constant anodal current of 100 msec duration through the membrane. A further prolongation of the anodal current up to 10 sec did not enhance the excitability of the fibre. Only when the polarizing current lasted several minutes an occasional fibre, previously inexcitable responded with a spike to the cathodal shock. The drawback of using a steady current lasting for several minutes was that only a few microelectrodes passed this amount of charge.

In confirmation of our previous findings (Albuquerque and Thesleff 1967) we observed that in a dose of 1—2 μg/ml PhC consistently after 1 hr abolished action potential generation.

The mean values for the amplitude of the action potential, its rate of rise and the threshold potential for its generation are shown in Table I. Removal of PhC by washing the preparation for 1—2 hrs with basic Ringer solution did not restore excitability as shown in Table I and Fig. 1 A—B. When the external calcium concentration was raised from 2 mM (normal calcium) to 15 mM (high calcium) the muscle membrane again became excitable and action potentials were recorded (Fig. 1 C). Recovery of spike generation with high calcium was observed even after 2 hrs of incubation with 1.5 μg/ml of PhC. Since 15 and 30 mM calcium solutions were about equally effective in restoring excitability was the firstmentioned concentration, at which the difficulty of calcium precipitation in the phosphate buffer was not encountered, employed in all experiments. When the calcium concentration was brought back to normal (2 mM) action potential generation was again abolished. Similarly when the muscle was incubated with 1—2 μg/ml of PhC in the presence of high calcium the action potential generation was maintained but the spike amplitude and its rate of rise were reduced. A subsequent change to a solution containing normal calcium and no PhC made the fibres rapidly inexcitable. With 3 μg/ml of PhC in high calcium, spike generation was abolished in most of the fibres (Fig. 2).

In high calcium, prior to incubation with PhC, the threshold for excitation was increased by about 10 mV while the amplitude and the rate of rise of the action potential remained relatively unchanged as compared to the values obtained in basic Ringer solution (Table I). Following incubation with PhC high calcium restored the excitability of the muscle membrane and action potentials were recorded in 45 out of the 50 muscle fibres investigated. The amplitude of the action potential was reduced by about 10 mV and the threshold for its generation increased. The rate of rise of the action potential declined by about 40 per cent as compared to the value obtained in high calcium prior to the incubation with PhC (Table I and Fig. 3).

TABLE I The resting membrane potential, input resistance, time constant of the membrane (τm) and the action potential are the mean values ± S.D. The figures within parenthesis are the numbers of fibres studied

	Resting membrane potential mV	Input resistance $\times 10^{6}$	τm msec.	Action potential		
				Threshold mV	Amplitude mV	Rat of rise V/sec.
Basic Ringer	73 ± 5.5 (189)	3.7 ± 0.4 (38)	2.6 ± 0.6 (29)	22 ± 6.6	86 ± 15.3 (153)	329 ± 86 (116)
$[Ca]_o$ 15 mM	76 ± 7.0 (62)	4.0 ± 0.5 (17)	2.6 ± 0.4 (15)	32 ± 7.0	86 ± 14.8 (41)	297 ± 38 (19)
$[Ca]_o$ 15 mM $[K]_o$ 10 mM	63 ± 2.2 (27)	2.4 ± 0.3 (12)	1.5 ± 0.3 (12)	31 ± 5.1	62 ± 6.9 (16)	171 ± 28 (16)
$[Ca]_o$ 15 mM $[K]_o$ 15 mM	56 ± 3.8 (31)	—	—	48 ± 9.1	55 ± 10.8 (31)	43 ± 4 (13)
$[Ca]_o$ 15 mM $[Rb]_o$ 5—6 mM	66 ± 4.1 (23)	—	—	31 ± 4.4	70 ± 7.0 (23)	149 ± 59 (15)
$[Ca]_o$ 15 mM $[Cs]_o$ 10 mM	64 ± 5.2 (28)	—	—	33 ± 6.6	66 ± 10.5 (26)	98 ± 42 (21)
PhC 1—2 µg/ml Basic Ringer	55 ± 11.2 (107)	2.1 ± 0.7 (26)	0.9 ± 0.3 (22)		5/87	—
Basic Ringer	59 ± 13.8 (52)	1.4 ± 0.6 (11)	0.9 ± 0.5 (11)		4/47	—
$[Ca]_o$ 15 mM	77 ± 6.3 (83)	2.7 ± 0.3 (23)	0.9 ± 0.3 (21)	40 ± 8.0	75 ± 15.0 (45)	178 ± 49 (26)
$[Ca]_o$ 15 mM $[K]_o$ 10 mM	66 ± 5.2 (43)	2.2 ± 0.5 (17)	0.7 ± 0.3 (18)		0/25	—
$[Ca]_o$ 15 mM $[K]_o$ 15 mM	54 ± 4.3 (24)	—	—		0/24	—
$[Ca]_o$ 15 mM $[Rb]_o$ 5—6 mM	71 ± 7.0 (19)	—	—		3/19	—
$[Ca]_o$ 15 mM $[Cs]_o$ 10 mM	56 ± 13.0 (10)	—	—		1/10	—

The number of fibres in which action potentials were elicited out of the total number of fibres investigated.

As shown by Albuquerque and Thesleff (1967) PhC in doses higher than 3 µg/ml caused visible damage to the membrane of innervated rat muscles while chronically denervated muscles tolerated more than ten times the amount of PhC. The action potential generating mechanism was blocked in all muscles whether innervated or denervated, by PhC in a dose of 1.5 µg/ml. The possibility that high calcium could restore the generation of action potentials after high doses of PhC was tested in experiments in which chronically denervated extensor or soleus muscles of the rat were incubated for 1 hr in 12.5, 25 and 50 µg/ml of PhC. The records in Fig. 4 show that

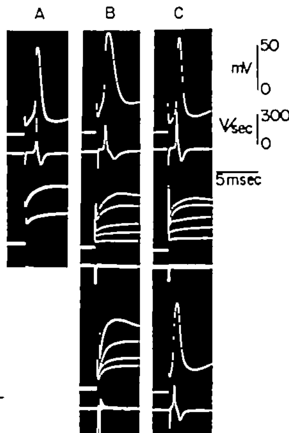


Fig. 1 Intracellular records from the extensor digitorum longus (A C) and soleus (B) muscles showing the changes in membrane excitability produced by 1.5 μg/ml of PhC and subsequent washing of the preparation with basic Ringer (A, B) and with high $[Ca]_o$ Ringer (C) The upper set of records are the action potentials and their first derivatives recorded in basic Ringer Middle set of records represent the effects produced after 1 hr of incubation with PhC when no spikes could be elicited. The lower set of records show that the generation of action potentials did not return when the preparation was washed in basic Ringer (A and B) but that partial recovery of excitability occurred in high calcium Ringer (C) The resting membrane potentials of the fibres were respectively for A = 76 60 56 mV B = 73 55 66 mV and C = 79 44 84 mV

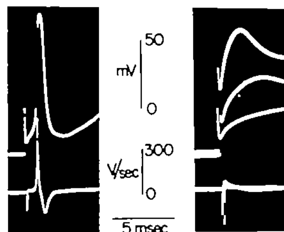


Fig 2. Effect of 1 hr of incubation with 5 μg/ml of PhC in the presence of high $[Ca]_o$ on action potential generation in the extensor digitorum longus muscle (right record) The left record is the control in high calcium Ringer without PhC. The resting membrane potentials were respectively 77 and 75 mV

high calcium partially restored spike generation in the muscle incubated with 1.5 although it failed when 25 or 50 μg ml of PhC had been used.

The nature of the action potential recorded in high calcium following incubation with PhC, was investigated by reducing the external sodium concentration and by

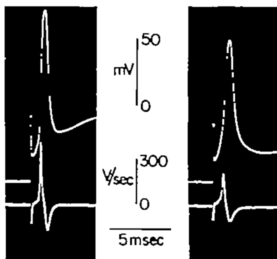


Fig. 3 Records of action potentials and their first derivatives in the extensor digitorum longus muscle in high $[Ca]_o$ before (left record) and after (right record) the muscle being incubated for one hour in 1.5 µg/ml of PhC. Note in the right record the reduction in amplitude and the rate of rise of the action potential. The resting membrane potentials were respectively 80 and 78 mV

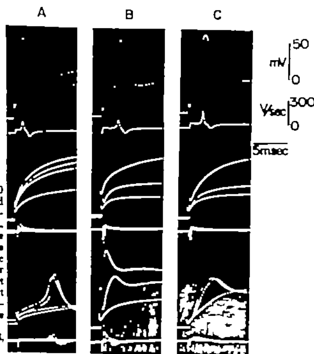


Fig. 4 Effect of 12.5, 25 and 50 µg/ml of PhC (records A, B and C) on spike generation in chronically denervated soleus muscles. The upper set of records are the controls in basic Ringer. The middle set of records depicts the effect of PhC produced after 1 hr of incubation, and the lower set of records demonstrates the effect of high $[Ca]_o$. The resting membrane potential of the fibres were respectively A — 65, 50, 60 mV, B — 60, 40, 50 mV and C — 58, 37, 54 mV

the application of tetrodotoxin (Fig. 5). This figure illustrates that high calcium did not restore spike generation when the external sodium concentration was reduced to 40 mM and 160 mM sucrose added to the medium. Furthermore tetrodotoxin, an agent which specifically blocks the sodium conductance change (Kao 1966), in a concentration of 1 µg/ml, prevented the generation of spikes in high calcium. Con-



Fig. 5 Reduced [Na]$_o$ (two thirds of sodium chloride replaced by sucrose) (Lower record in A) and 1 µg/ml of tetrodotoxin (Lower record in B) prevent recovery of spike generation in high [Ca]$_o$. The upper records are control action potentials in basic Ringer. The middle records show the block of spike generation following 1 hr of incubation with 1.5 µg/ml of PhC. Resting membrane potential A — 77, 60, 86 mV; B — 73, 57, 75 mV

sequently the action potential after the treatment with PhC and subsequent restoration by high calcium involves a sodium current as in normal muscle fibres.

Two other bivalent ions were tried in order to reproduce the effects of high calcium. Strontium in a concentration of 15 mM had qualitatively similar effects as high calcium and was able to repolarize the fibres and to restore spike generation in PhC treated muscles. On the other hand, 15 to 45 mM of magnesium caused no recovery of excitability and neither were the fibres repolarized after the effect of PhC. On the contrary the resting membrane potential fell further in the presence of 30 and 45 mM magnesium. In the presence of high calcium 15 mM of magnesium had no effect on the recovery caused by the former ion.

Potassium, caesium and rubidium have long been known to antagonize and/or displace calcium in some of its actions on cell and artificial lipid membranes (Ringer 1884, Krogh, Lindberg and Schmidt-Nielsen 1944, Relman 1956, Schmidt and Stämpfli 1957, Sjodin 1959, Goldman 1964). Experiments were therefore made with high calcium Ringer containing either 10 mM potassium, 15 mM potassium, 10 mM caesium or 6 mM rubidium. These concentrations of the univalent cations produced in normal muscle a reduction in the height and the rate of rise of the action potential (see Table I) but in no fibre did they abolish the spike. However following treatment with PhC and the subsequent recovery of the spike with high calcium their effects were striking. As shown in Fig. 6 and Table I potassium, caesium and rubidium in practically all the fibres abolished action potential generation.

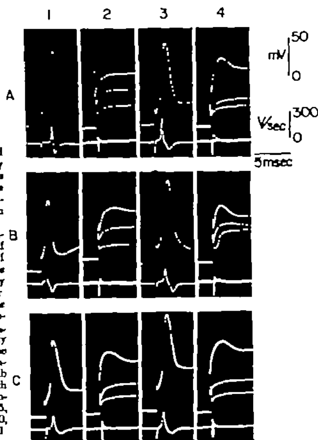


Fig. 6. Effects of $[K]_o$, $[Rb]_o$, and $[Cs]_o$ on the membrane excitability of the extensor digitorum longus muscle before and after blockade of spike generation by the incubation of the muscles for 1 hr with 1.5 μg/ml of PhC. The traces A-1, B-1, C-1 are respectively the controls in high $[Ca]_o$ with 10 mM KCl, 6.3 mM RbCl and 10 mM CsCl. A-2, B-2, and C-2 shows the block of the spike generation by 1.5 μg/ml of PhC in basic Ringer. A-3, B-3, and C-3 illustrate the partial recovery of the action potential after 1 hr in high $[Ca]_o$. A-4, B-4, C-4 show respectively the disappearance of membrane excitability when the aforementioned concentrations of K, Rb and Cs were added to the high calcium Ringer. Resting membrane potential: A 1—4 = 65, 57, 79, 67 mV; B 1—4 = 62, 60, 81, 63 mV; C 1—4 = 60, 54, 81, 61 mV.

Resting membrane potential and input resistance

Since an intimate relationship exists between the excitability, the resting potential and the ionic permeability of the cell membrane experiments were made to see if the observed excitability changes were accompanied by alterations in the resting membrane potential and the input resistance of muscle fibres.

Incubation with PhC in a dose of 1—2 μg/ml had a variable effect on the resting membrane potential and the ionic permeability recorded as input resistance of muscle fibres. As shown by the mean values in Table 1, the general effect after 1 hr incubation with PhC was a fall in the membrane potential from 73 mV to 55 mV and a reduction by about 40 per cent of the input resistance. In our previous investigation (Albuquerque and Thesleff 1967) these amounts of PhC caused no or only a small reduction of the input resistance and the membrane potential. However, when the dose of our present PhC preparation was reduced to 1 μg/ml the effects resembled those previously obtained with 1.5 μg/ml. Besides, in the present investigation incubation times were always longer than 1 hr while they in our previous study were at most 60 min.

Removal of PhC by washing the preparation with basic Ringer solution had no effect on the resting membrane potential and even further reduced the input resistance (Table I). In the normal muscle high calcium did not affect the resting membrane potential nor the input resistance while in the preparation after incubation with PhC repolarization of the membrane to 77 mV occurred. Only a partial recovery of input resistance was observed (Table I). Incubation with 3 µg/ml of PhC together with high calcium did not depolarize the fibres (Fig. 2). When the calcium concentration was reduced to normal the resting membrane potential fell to values similar to those previously recorded in PhC and the input resistance remained low.

In solutions containing high calcium the addition of potassium (10 or 15 mM), caesium (10 mM) or rubidium (6 mM) reduced the resting membrane potential by 10—20 mV to 55—70 mV irrespective of whether it was a normal muscle or a muscle following PhC treatment (Table I). On input resistance only the effects of 10 mM potassium were examined. From the mean values presented in Table I it is noted that potassium, caesium and rubidium in normal muscles reduced the resting membrane potential and the input resistance of muscle fibres by about the same amount as did incubation with PhC in basic Ringer solution. A marked difference between the conditions was that before incubation with PhC action potentials were recorded in all the fibres while after the action of PhC spike generation was blocked. Furthermore, following incubation with PhC, the univalent ions prevented high calcium from restoring the resting membrane potential and the excitability of the muscle fibres.

In connection with the recording of the input resistance the electric time constant of the membrane was measured (Table I). Incubation with PhC reduced the time constant to 1/3 of its normal value while the input resistance fell by only 40 per cent. In high calcium, which restored membrane excitability and repolarized the fibres, the electric time constant as well as the input resistance of muscle fibres remained low.

Discussion

The investigation has shown that incubation with PhC abolished spike generation. When the calcium ion concentration of the external solution was increased from 2 to 15 mM membrane excitability returned and was maintained as long as the calcium concentration remained high. Despite that the excitability of muscles incubated with PhC was restored by calcium this ion was unable to bring the cell membrane back to normal. The input resistance of muscle fibres remained at 2/3 and the electric time constant at 1/3 of their control values. Neither did excitability completely recover since the rate of rise and the amplitude of the action potential were reduced as compared to untreated muscles. The changes produced in the muscle membrane by incubation with PhC were therefore of irreversible nature and calcium could only functionally restore some of the membrane properties.

Recovery of membrane excitability in the presence of high calcium could be explained by an effect of the ion on the potential and the input resistance of the resting

membrane and/or by an action on those membrane tructures which control the movements of sodium and potassium during the action potential (Frankenhaeuser and Hodgkin 1957)

When the calcium concentration, following incubation with PhC, was raised to 15 mM the muscle membrane polarized from 55 to 77 mV The possibility that this repolarization was the sole cause of the recovery of excitability is not likely since in solutions w th normal calcium anodal polarization of the membrane to 70—80 mV was unable to restore spike generation. Furthermore, when the muscle was incubated with PhC in a dose of 3 μg ml together with high calcium pike generation was abolished but the resting membrane potential remained above 70 mV However in view of the known dependence of the sodium conductance change upon the level of membrane polarization (Frankenhaeuser and Hodgkin 1957) there can be little doubt that the observed increase in the resting potential contributed to the recovery of excitability

Incubation with PhC increased the ionic permeability of the muscle membrane as shown by the fall in the input resistance of fibres. In high calcium action potentials were recorded but the input resistance remained reduced. Similarly 10 and 15 mM of potassium of 10 mM caesium, when added to the normal muscle, reduced the input resistance by about 40 per cent but the membrane maintained its excitability Keeping the input resistance at the normal level is therefore not a prerequisite for action potential generation nor for the repolarization following PhC in high calcium.

The tructures which control the passive transport of sodium and potassium across the membrane ha e been suggested to be negatively charged phosphate groups of certain phospholipid molecules acting as ion exchange sites (Goldman 1964) As discussed by Albuquerque and Thesleff (1967) and Thesleff and Albuquerque (1967) it is possible that the observed effects of PhC on membrane excitability are the result of the lipase activity of the preparation and therefore secondary to hydrol ysis of membrane phospholipids. The irreversibility of the effects prod ced by PhC in muscle, provides additional support for an enzymic action of PhC on the cell membrane.

Evidence from biological and artifical lipid membranes fa our the iew that calcium ions are electrostatically bonded to dipolar complexes of phospholipids (Kimizuka and Koketsu 1962, Woolley and Campbell 1962. Le h and Tobias 1964 Rojas and Tobias 1965) It has also been suggested that the al um ion b h dration ire or possibly by its condensing effect on phosphol pid head molecules ts as gat for the control of passive ion transfer (Lett m t al 1964 Tobias t al 1962, T bias 1964)

With these assumptions in mind one ma peculate that f llowing incubation w th PhC, high calcium restores membrane excitabilit b one or both of th following mechanisms The condensing ffect of calcium ion on elementar units in the mem brane could account for the repolarization of the muscle f bre nd thereby be an implement to the restoration of pike generation Condensing of membrane tructures could possibly also functionally restore action potential generation damaged by PhC

Another mechanism would be that calcium ions by combining with carrier groups in the membrane, not previously attacked by PhC enhanced their efficacy as ion transport sites and thereby enabled spike generation. This suggestion is similar to that proposed to explain the interaction between calcium and local anaesthetics in the voltage clamped squid giant axon (Shanes *et al.* 1959).

The observation that potassium, caesium and rubidium in the presence of high calcium abolished action potential generation after but not prior to the incubation with PhC is presumably not the result of differences in the resting membrane potential since these were insignificant. Possibly these alkali ions, as suggested by Mullins (1960, 1961) and Rosenblum, Stein and Story (1966) compete with calcium for membrane structures in which action potential sites are located and thereby prevent calcium from controlling the sodium and potassium permeability. If the number of action potential sites in th membrane were reduced by the action of PhC it could be expected that these concentrations of the univalent ions brought spike generation to a subcritical level.

The study was supported in part by research grants from the Swedish Medical Research Council (14 738-02) Stockholm, Sweden, and the Medical Faculty University of Lund, Lund, Sweden. Expert technical assistance was provided by Miss B. Hansson.

References

Aceves, J. and X. Machne. The action of calcium and of local anesthetics on nerve cells, and their interaction during excitation. *J. Pharmacol. exp. Ther.* 1963 *140* 138—148.

[illegible] E. X. and S. Thesleff. Influence of phospholipase C on some electrical properties of the skeletal muscle membrane. *J. Physiol.* (Lond.) 1966 *190* 123—137.

[illegible] I. A. and A. R. Martin. Membrane constants of mammalian muscle fibres. *J. Physiol.* (Lond.) 1959 *147* 450—45

Feinstein, M. B. Reaction of local anesthetics with phospholipids—a possible chemical basis for anesthesia. *J. gen. Physiol.* 1964 *48* 357—374.

Frankenhaeuser, B. and A. L. Hodgkin. The action of calcium on the electrical properties of squid axons. *J. Physiol.* (Lond.) 1957 *137* 218—244.

Goldman, D. E. A molecular structural basis for the excitation properties of axons. *Biophys. J.* 1964 *4* 167—188.

Goldman, D. E. Gate control of ion flux in axons. *J. gen. Physiol.* 1965 *48* 75—77.

Kimizuka, H. and K. Koketsu. Binding of calcium ion to lecithin film. *Nature* (Lond.) 1962 *196* 995—996.

Koketsu, K., R. Kitamura and R. Tanaka. Binding of calcium ions to cell membrane isolated from bullfrog skeletal muscle. *Amer. J. Physiol.* 1964 *207* 509—512.

Krogh, A., A. L. Lindberg and B. Schmidt-Nielsen. The exchange of ions between cells and extracellular fluid. II. The exchange of potassium and calcium between the frog heart muscle and the bathing fluid. *Acta physiol. scand.* 1944 *7* 221—237.

Leitch, G. J. and J. Tobias. Phospholipid—Cholesterol membrane model: effects of calcium, potassium or protamine on membrane hydration, water permeability and electrical resistance. *J. cell. comp. Physiol.* 1964 *63* 225—232.

Lettvin, J. Y., W. F. Pickard, W. S. McCulloch and W. Pitts. A theory of passive ion flux through axon membranes. *Nature* (Lond.) 1964 *202* 1338—1339.

Mullins, L. J. The penetration of some cations into muscle. *J. gen. Physiol.* 1959 *42* 817—829.

Mullins, L. J. An analysis of pore size in excitable membranes. *J. gen. Physiol.* 1960 *43* Suppl. 1 105—117.

Mullins, L. J. The macromolecular properties of excitable membranes. *Ann. N. Y. Acad. Sci.* 1961 *94* 390—404.

Relman, A. S The physiological behavior of rubidium and cesium in relation to that of potassium. *Yale J Biol. Med.* 1956. 29 248—262.

Ringer, S., An investigation regarding the action of rubidium and caesium salts compared with the action of potassium salts on the ventricle of the frog's heart. *J Physiol.* (Lond) 1883 4 370—378.

Rojas, E. and J M. Tobias, Membrane model, association of inorganic cations with phospholipid monolayers. *Biochim. biophys. Acta.* (Amst.) 1965. 94 394—404

Rombalski, L, A. A. Stein and J L. Story Effects of cesium, potassium and rubidium on development of tension in isolated human uterine muscle. *J Pharmacol. exp Ther* 1966. *152* 231—234.

Schmidt V H. and R. S Amstel, Die Depolarisation durch Calcium—Mangel und ihre Abhängigkeit von der Kalium—Konzentration. *Helv physiol. pharmacol. Acta.* 1957 *15* 200—211

Shah, D O. and J H. Schulman Binding of metal ions to monolayers of lecithins, plasmalogen, cardiolipin and dicetyl phosphate. *J Lipid Res* 1965. 6. 341—349

Shanes, A. M Electrochemical aspects of physiological and pharmacological action in excitable cells. I The resting cell and its alteration by extrinsic factors. *Pharmacol. Rev* 1958a. *10* 59—164

Shanes, A. M., Electrochemical aspects of physiological and pharmacological action in excitable cells. II. The action potential and excitation. *Pharmacol. Rev.* 1958b. *10* 165—273

Shanes, A. M W H. Freygang, H. Grundfest and E. Amatniek, Anesthetic and calcium action in the voltage clamped squid giant axon. *J gen. Physiol.* 1959 *42* 793—802.

Sjödin, R., Rubidium and cesium fluxes in muscle as related to the membrane potential. *J gen. Physiol.* 1959 *42*. 983—1003.

Taylor, R. E. Effect of procaine on electrical properties of squid axon membrane. *Amer J Physiol.* 1959. *196* 1071—1078.

Thesleff S. and E. X. Albuquerque, The cholinergic receptor systems of denervated striated muscle. *Ann. N.Y Acad. Sci.* 1967 In press.

Tobias, J M., Experimentally altered structure related to function in the lobster axon with an extrapolation to molecular mechanisms in excitation. *J cell comp Physiol.* 1958. *52* 89—126.

Tobias, J M., D. P Agin and R. P Wlodawer, Phospholipid-cholesterol membrane model—control of resistance by ions or current flow *J gen. Physiol.* 1962 *45* 989—1001

Tobias, J M A chemically specified molecular mechanism underlying excitation in nerve hypothesis. *Nature* (Lond.) 1964. *203* 13—17

Woolley D W and N K. Campbell, Tissue lipids as ion exchangers for cations and the relationship to physiological processes. *Biochim biophys Acta* (Amst.) 1962 *57* 384—385.

Acta physiol. scand. 1968. 72. 322—326

From the Institute of Physiology and Medical Biophysics and the Department of Paediatrics, University of Uppsala, Sweden

Formation of Bilirubin in the Dog Spleen

By

L. BÄCKLUND and M. MICHAËLSSON

Received 26 June 1967

Abstract

BÄCKLUND, L. and M. MICHAËLSSON. *Formation of bilirubin in the dog spleen.* Acta physiol. scand. 1968. 72. 322—326.

No reliable informations on the normal plasma bilirubin contents in dog are given in the literature. In the present investigation physiological sampling procedure and modern analytic technique was used and data of blood from splenic vein, the abdominal aorta and central vein were collected. The significance of the findings is discussed. In order to obtain information on the site of formation of bilirubin erythrocyte destruction was induced by injection of nicotinic acid and stored blood. On the basis of the results a bilirubin releasing mechanism in the spleen is concluded.

Since a long time it is known from a great number of investigations that the liver can form bilirubin. Most likely this is true about the spleen too and probably also for the whole reticulo-endothelial system. The formation of bilirubin is not exclusively connected to any particular cellular activity. For example, bilirubin can be identified in extravasated blood. For further discussion and references see With (1960).

The present investigation deals with the differences in bilirubin concentration in venous blood from the spleen, central venous blood and arterial blood during normal conditions in dog and after destruction of red cells induced by nicotinic acid or storage of blood.

The investigation had two purposes. The first one was to study the site of formation of bilirubin in dog. The second one was to collect data on the normal plasma bilirubin concentration in dog. This seems to be indicated because of the fact that many studies on bile pigment still are made in dogs. Furthermore according to the literature the normal dog plasma contents of bilirubin are uncertain. For instance, Varela Fuentes and Munilla (1934) and Fahrak (1948) could not prove any bilirubin in dog blood serum. This contradicts the reports by Lopez Garcia, Zelasco and Pedace (1943).

Material and methods

19 grown up mongrel dogs were used in the experiments. The weight varied from 10 to 33 kg. General anesthesia was induced by i.v. injection of sodium pentobarbitone (Veterinary Nembutal® Abbott) in doses of 30 mg/kg body weight. In order to obtain blood samples polythene catheters were inserted into the femoral vessels in one side. The arterial catheter was placed with its tip in the abdominal aorta while the tip of the vein catheter was situated in the inferior vena cava or in the right atrium. In the femoral vein in the other side a further catheter was inserted. Through this catheter injections were made (solution of nicotinic acid, stored citrate blood, additional anesthetic). The abdomen was opened with a mid-line incision and splenic venous blood was obtained after puncture or transection of a splenic vein. After each sampling the abdomen was closed again. All blood samples disregarding the origin were collected in heparinized tubes. The samples varied between 2 and 10 ml depending on the size of the animal.

The first part of the series included 13 dogs from which only one blood sample was taken from the artery, the central vein and the splenic vein respectively. From the rest of the cases (6 dogs) further samples were taken after i.v. injection of nicotinic acid (5 % solution, 1 mg/kg body weight). 4 of these 6 dogs were also given stored citrate blood (20—50 ml i.v.) 70—140 min after the injection of nicotinic acid. The citrate blood was collected from other (intact) dogs and had been stored at +4° C in citrate solution (ACD-Vitrum® ACO) for 14—24 days.

Blood samples for analyses were taken before as well as after the injection of nicotinic acid resp. stored blood. 30 min after each injection blood was collected from the artery and the central vein. 60 min after the injections samples were taken from all studied types of vessels. In one case samples were also taken after 120 min in order to study the duration of the effect of the nicotinic acid.

The analyses performed were determinations of the plasma contents of bilirubin and free hemoglobin. The determinations of bilirubin were made with a modified Jendrassik-Gróf-Vossilla method (Michaëlsson 1961). According to this method bilirubin values lower than 0.12 mg per 100 ml are not significantly higher than zero values. The plasma hemoglobin contents were determined according to Crosby and Furth (1956).

Results

The mean value of the plasma bilirubin concentration in splenic venous blood was 0.10 mg per 100 ml, range 0.0—0.28. Only in 5 of the 19 cases values higher than 0.12 were found. The mean value of arterial blood was 0.06 mg per 100 ml, range 0.0—0.17. In 2 cases the concentration was higher than 0.12. The mean value of central venous blood was 0.08, range 0.0—0.24 with 4 values higher than 0.12. Only in 1 dog the bilirubin contents were higher than 0.12 mg per 100 ml in blood from all the studied types of vessels.

The plasma contents of bilirubin and hemoglobin before and after injection of nicotinic acid are shown in Fig. 1 and Fig. 2. A slight but significant increase of the bilirubin contents in splenic vein was obtained but no differences could be found in arterial blood or central venous blood. The case in which samples also were taken 120 min after the injection of nicotinic acid showed no significant changes in the concentrations after the 60th min although a very slight decrease was found.

The effects of injected stored citrate blood are demonstrated in Fig. 3 and Fig. 4. The concentration of plasma bilirubin in the splenic vein rose significantly in 3 of the 4 dogs studied. The increase of plasma bilirubin concentration in the other vessels was, however, not significant. The concentration of plasma hemoglobin was usually higher after the injection in all the types of blood investigated.

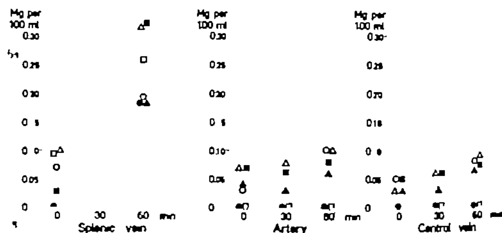


Fig. 1 Each symbol represents data collected from a given dog. The same symbol always represents the same dog in the different pictures.

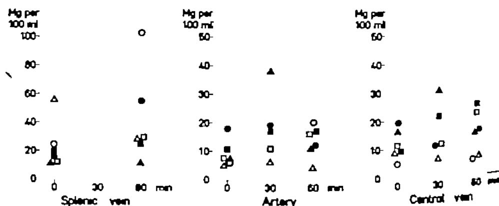


Fig. 2. *Cf.* Fig. 1 Note the different scales for concentration.

Attempts were made to study blood from other organs known or supposed to produce bilirubin, *i.e.* the liver and the bone marrow. It was found to be very difficult to obtain blood from these tissues in sufficient quantities without admixture of blood from other organs. It was not possible to get any reliable results.

Discussion

The low values obtained for plasma bilirubin concentration in dog are, as a rule, not significantly higher than zero values in any vessel studied in this report. This is in agreement with Dahlgren (1964) who used dog venous blood and the same analytic technique. With 30 dogs Dahlgren got a mean value of 0.12 mg bilirubin

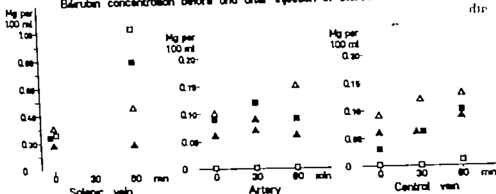


Fig. 3 *Cf* Fig. 1 Note the different scales for concentration.

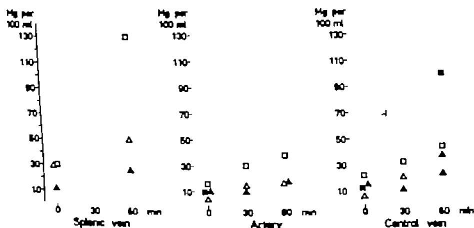


Fig. 4 *Cf* Fig. 1

per 100 ml plasma. As a matter of fact the plasma bilirubin levels in dog re so low that even the method here used is less suitabl for the determination. Other methods for the determination of plasma bilirubin ha e the same disadvantages As compared to h man plasma bilirubin concentration the dog plasma concentration is mall Th ould be explained in several ways. The bilirubin production rat could be lower or the capacity in conjugating bilirubin could be greater thus indicating a different bilirubi metabolism in dogs. However the difference in normal plasma bilirubin contents between man and dog means that results from dog studies are not directly pplicable to man.

The slight but significant ncrease in bilirubin contents in splenic enous blood fter injection of nicotinic acid indicates that this drug has the same effects on dog

as has been described on man (Gydell 1960 a) The main effect seems to occur within 60 min the only case studied for 120 min showed no further elevation of the bilirubin level. This is in agreement with the findings in man, where the effect is obvious after 30 min and mainly has occurred during the first hour after the injection (Gydell 1960 b)

The rather marked increase in plasma hemoglobin concentration after injection of stored blood can be explained in different ways. First, the stored blood itself had reasonably high contents of plasma hemoglobin. Secondly an increased rate of erythrocyte destruction is likely to occur in the spleen as well as in other organs and in circulating blood.

An observation indicating that the hemoglobin transport mechanism could be the same in dog as has been suggested for man is the plasma haptoglobin concentration. In a separate series of 10 intact dogs the venous blood contents of haptoglobin were determined with the method described by Connell and Smithies (1959) A mean value of 116 mg per 100 ml serum was found. The range was 48—189 mg per 100 ml which is within the range of normal human values.

No difference in plasma bilirubin concentration between splenic venous blood, arterial blood and central venous blood respectively could be found in intact dogs. Thus it is not possible to prove that the spleen can produce bilirubin under normal conditions. The higher levels of plasma bilirubin in splenic blood after injection of nicotinic acid as well as stored blood indicates that the spleen at least under such circumstances can produce bilirubin. The effect of nicotinic acid, however is not so marked that the bilirubin concentration increases in the systemic blood vessels.

References

CONNELL, G. E. and O. SMITHIES, Human haptoglobins: estimation and purification. *Biochem. J.* 1959. *72*. 115—126.

CROSBY, W. H. and F. W. FURTH, A modification of the benzidine method for measurement of hemoglobin in plasma and urine. *Blood* 1956. *11*. 380—383.

DAHLGREN, S. The afferent loop syndrome. *Acta chir. scand.* 1964. Suppl. 327

FAHRAK, N. The problem of jaundice. Bilirubinaemia in warmblooded animals with its bearing on natural immunity and its defensive role in infections. *J. Egypt. med. Ass.* 1948. *31* 522—548.

GYDELL, K., Hyperbilirubinemia and hypersideremia following intravenous injection of stored blood, nicotinic acid and hemoglobin solution. *Acta med. scand.* 1960. *166* 433—443.

GYDELL, K. Transient effect of nicotinic acid on bilirubin metabolism and formation of carbon monoxide. *Acta med. scand.* 1960 b. *167* 1—11

LOPEZ GARCIA, A. J. F. ZELASCO and E. A. PEDACE, Ligadura experimental del coledoco y estudio de la colemia y de la coluria en diversas especies animales. *In. Inst. Invest. fis. Pat. hum.* (*B. Aires*). 1943. *5*. 13—58.

MICHAËLSSON, M., Determination of bilirubin in serum and urine. *Scand. J. clin. Lab. Invest.* 1961. Suppl. 56.

VARELA FUENTES, B. and A. MUNILLA, Bilirubine du sérum de quelques vertébrés. *C. R. Soc. Biol.* (*Paris*) 1934. *117* 555—557

WITH, T. *Biologie der Gallenfarbstoffe*. Georg Thieme Verlag, Stuttgart, 1960.

Acta physiol. scand. 1968. 72. 327—330

From the Department of Physiological Chemistry University of Lund, Sweden

The Distribution of Fatty Acids between Serum Albumin and Red Cell Ghosts

By

GÖRAN GÖRANSSON

Received 28 June 1967

Abstract

GÖRANSSON, G *The distribution of fatty acids between serum albumin and red cell ghosts.* Acta physiol. scand. 1968. 72 327—330.

The chemical composition of red cell ghosts are reported. Incubations with red cell ghosts and labeled fatty acids bound to albumin have been carried out. The results show that long and saturated acids are bound to a greater extent to the cell membranes than short and unsaturated acids. With an increasing mole ratio fatty acids/albumin an increasing percentage of oleic acid was transfered to the cell membranes. It is pointed out that the same tendency was noted earlier in experiments with serum, lipo-proteins and fatty acids bound to albumin.

The uptake of free fatty acids from serum by cells takes place in two steps (Fillerup Migliore and Mead 1958) First the fatty acids are distributed between the serum proteins and the cell walls, then there is a metabolism of the fatty acids that requires the energy production in the cell to be intact. The present experiments have been performed in an attempt to investigate the first part of the uptake of fatty acids. Labeled fatty acids have been incubated with human serum albumin and red cell ghosts and the distribution of label has been studied.

Materials and methods

Red cells ghosts were prepared from blood which was obtained from normal male rats (300 g) 1 volume of 10 % Na-citrate per 100 volumes of blood was used to prevent blood clotting. The red corpuscles, obtained by centrifugation at 4000 rpm for 10 min, were washed three times with saline and then hemolysed with 20 volumes of distilled water HCl was added to pH 6 and the temperature was lowered to 4 C This was done to facilitate the dissociation of the hemoglobin from the cell walls (personal information from dr Löw The blood bank, Hospital of Lund, Lund) After hemolysis over night with continuous stirring, the red cell ghosts were washed 8 times with 1 % NaCl-solution at 4 C (pH 6) The red cell ghosts were then suspended in saline at 25 C at pH 7 and placed in incubation flasks. Before the incubations, which took place at 37 C and for 30 min (which was enough time for equilibration) the red cell ghosts were suspended in 4.5 % albumin (Kabi 1067 Human serum albumin dissolved in saline) To fixed amount of ghosts enough serum albumin solution was added so that the volume corresponded to that of the blood from which the red cell ghosts had prepared minus the volume of the ghosts.

Some of the red cell ghosts were f eeze-dried and used for determination of the chemical composition of the ghosts. The methods that w re used for extraction, separation and quantitative determination of the lipid were those described earlier (Göransson and Olivecrona 1964). The labeled and non-labeled fatty acids, which were complexed with albumin before the incubations, were the same as in earlie experiments (Göransson 1965 a). The incubation medium was prepared in the same way as the injection solutions used by Göransson and Olivecrona (1964).

The amount of radioactivity taken up by the ed cell ghosts was determined by calculation of the differenc between the amount added to the incubation medium and the amount left in the supernatant after incubation and centrif gation at 4000 rpm for 10 min. Radioactivity was determined as described by Göransson and Olivecrona 1964.

Results

The chemical composition of the red cell ghosts is given in Table I. About 12.5 % of the dry weight was made up of lipid, 86 of the lipid was phospholipids, 13 % was free cholesterol and only 1 was glyceride. Cholesterol esters and free fatty acids could not be detected with the present methods because they were present in too small amounts.

Table II shows results from experiments in which increasing amounts of oleic acid bound to a fixed amount of albumin were incubated with red cell ghosts. To different incubation flasks 0.02, 5 or 10 μeq of oleic acid per ml of 4.5 % albumin sol. were added. The results suggest that with increasing amount of oleic acid a higher percentage of labeled acid was bound to ghosts.

TABLE I. Composition of red ell ghosts prepared from rat blood

Total amount of red cell ghosts	Lipid mg	Phospholipids mg	Cholesterol mg	Glycerides mg	Cholesterol esters mg
100	13.82	12.0	1.7	0.12	—
100	11.81	10.0	1.7	0.11	—
100	12.17	10.5	1.6	0.07	—
Mean	12.6	10.8	1.7	0.12	—

TABLE II. Results from periments in hich increasing amounts of oleic acid bound to constant amount of lbumin was ncubated w th red cell ghosts

C^{14}-oleic acid per ml of 4.5 albumin solution μeq	Unlabeled oleic acid per ml of 4.5 % albumin solution μeq	Labeled acid taken up[1] / Labeled acid not taken up
02	—	0.22
.02	5	0.54
02	10	0.73

Mean of two values

TABLE III. Results from incubations with different fatty acids (0.02 µeq per ml of 4.5 % albumin solution) and red cell ghosts

Acid	Amount of radioactivity that was taken up %	
Caproic	12	11
Lauric	13	16
Myristic	14	15
Palmitic	17	19
Stearic	26	32
Oleic	18	22

The results from experiments, in which saturated fatty acids with differing chain length were compared with each other and w th oleic acid, are seen in Table III To each incubation flask 0.02 µeq of fatty acid was added per ml of a 4 5 % albumin solution. The results indicate that long chain fatty acids had a greater tendency to become bound to the red cell ghosts than shorter acids. Oleic acid did not become bound to the red cell ghosts to the same extent as stearic acid.

Discussion

In the present experiments the chemical composition of the red cell membranes seems to corroborate what is known about cell membranes in general. Thus t has been shown (Hokin and Hokin 1965) that cell membranes are probably built p by protein and bimolecular layers of phospholipids and cholesterol. The red cell ghosts used in the present experiments are in fact lipoproteins nd this fact justifies a comparison between th present results and those from earlier experiments in which the distribution of fatty acids between serum lipo-proteins and albumin was studied. In both instances the distribution was studied after equil brium had been established. The results show that in both cases short and unsat rated cids were bound to higher degree to albumin than long and saturated acids It w also found in both cases that more fatty acid was bound to the lipoprotein (n the present experiments the cell membranes) with an ncreasing mole ratio of fatty acid t albumin The conclusion seems to be that serum lipoproteins and red cell membranes ha some properties in common as far as the binding of fatty acid, is concerned

Earlier experiments performed by Goransson and Oli ecrona (1965) and Goransson (1965 a, b, d, f and g) ha shown that short and nsatur ed f tt acids disappear more rapidly from the blood after injection than long and sat rated acids. This is true pit of the fact that the present experiments suggest that long chain saturated acids ha greater tendency to combine with cell membranes in itro. These results need not be contradictory as the experiments only giv an indication of the situation after equilibrium has been reached. Equilibrium could ery well ha

been reached after different times for different fatty acids. If it is assumed that the fatty acids have to pass a water phase to reach equilibrium it is logical that the more water-soluble acids should reach equilibrium first. This fact may explain why short acids in vivo disappear more rapidly from blood than long ones. A further explanation could be that short acids are metabolized (i.e. oxidized) within a shorter time than long acids as was shown by Göransson and Olivecrona (1965) and Göransson (1965 b, c, d, e, f and g)

References

Fillerup D L., J C. Mulmore and J F Mead, The uptake of lipoproteins by Ascites Tumor Cells. The fatty acid-albumin complex. *J Biol. Chem.* 1958. *233.* 98—101

Göransson, G The transport from of free fatty acids in rat serum. *Acta physiol. scand.* 1965 a. *64.* 269—274.

Göransson, G., The metabolism of fatty acids in the rat. III. Arachidic acid. *Acta physiol. scand.* 1965 b. *63.* 385—390.

Göransson, G The metabolism of fatty acids in the rat. IV Stearic acid. *Acta physiol. scand.* 1965 *63.* 425—427

Göransson G The metabolism of fatty acids in the rat. V Palmitoleic acid. *Acta physiol. scand.* 1965 d. *63.* 428—433.

Göransson, G The metabolism of fatty acids in the rat. VI. Arachidonic acid. *Acta physiol. scand.* 1965 e. *64* 1—5.

Göransson G The metabolism of fatty acids in the rat. VII Linoleic acid. *Acta physiol. scand.* 1965 f. *64* 204—210.

Göransson, G The metabolism of fatty acids in the rat. VIII Lauric and myristic acid. *Acta physiol. scand.* 1965 g. *64* 383—386.

Göransson, G and T Olivecrona, The metabolism of fatty acids in the rat. I. Palmitic acid. *Acta physiol. scand.* 1964 *62.* 224—

G and T Olivecrona, The metabolism of fatty acids in the rat. II Oleic acid. *Acta physiol. scand.* 196.. *63* 121—127

...akon, L. E. and M. R. Hokin, The Chemistry of Cell Membranes. *Scientific American* 1965. Oct. ..—86.

Acta physiol. scand. 1968. 72. 331—337
From the Institute of Physiology University of Oslo, Norway

The Inhibitory Effect of Various Phenols upon ATP induced Vasoconstriction in Isolated Perfused Rabbit Lungs

By

P. K. M. Lunde, B. A. Waaler and L. Walløe

Received 28 June 1967

Abstract

Lunde, P. K. M., B. A. Waaler and L. Walløe. *The inhibitory effect of various phenols upon ATP-induced vasoconstriction in isolated perfused lungs.* Acta physiol. scand. 1968. 72. 331—337

A spontaneously developing vasoconstriction in isolated perfused lungs and the vasoconstriction caused by arterially injected ATP (50 μg) were both inhibited by the addition to the perfusate of various phenols. The inhibitory effect upon ATP-induced vasoconstriction of phenol, the 3 isomers of cresol (mono-methyl-phenol) and the 6 isomers of xylenol (di-methyl-phenol) was compared on a rank scale. There was a definite pattern in the results. Methyl groups in either o- or p-position in relation to the hydroxy group increased the inhibitory effect of the phenol, whereas methyl group in m-position resulted in little inhibitory effect.

When working with an isolated blood-perfused rabbit lung preparation, Hauge, Lunde and Waaler (1966) observed that a marked vasoconstriction usually began to develop from 10 min to 2 hr after start of the perfusion. During the same period of the perfusion experiments the reaction of the pulmonary vascular bed to a standard dose of injected ATP changed from a small dilatation to a marked constriction. The spontaneously developing vasoconstriction as well as the vasoconstriction caused by injected ATP were both efficiently and somewhat surprisingly counteracted by addition to the perfusate of a commercial preparation of tri-cresol. When the three cresol isomers were tested separately m-cresol was found to have only a small inhibitory effect on the spontaneously developing vasoconstriction, whereas o- and p-cresol were more effective.

Preliminary investigations did further show that a certain dose of commercial tri-cresol caused a more marked inhibition of the ATP-induced vasoconstriction, than did an equal dose of either o- or p-cresol. A subsequent analysis of the commercial tri-cresol revealed that in addition to the cresols this preparation also contained some phenol and a certain amount of xylenols (di-methyl-phenols)

In the present work the inhibition of the ATP-induced pulmonary vasoconstriction by phenol and by the various cresol and xylenol isomers has therefore been evaluated and compared. A preliminary report of some of the results has been given earlier (Hauge *et al.* 1966 b).

Methods

Preparation. Isolated rabbit lungs were perfused with heparinized, homologous blood under conditions of constant volume inflow. About 250 ml of blood were used in each experiment. Positive pressure ventilation was used as described by Konzett and Rössler (1940). The pulmonary arterial pressure (PAp) and the ventilation overflow were recorded continuously. The left atrial pressure (LAp) was kept constant throughout an experiment at a level between 1 and 4 cm of water. Further details about the preparation with its perfusion and ventilation arrangements have been given in a previous paper (Hauge *et al.* 1966).

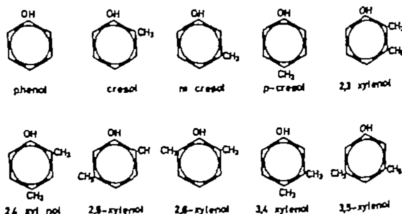
Drug addition. All drugs were dissolved in 0.9 % NaCl. ATP is rapidly inactivated in blood. Consequently ATP was injected into the pulmonary arterial tubing, near the pulmonary artery. Usually 50 μg of ATP in 0.5 ml were used as the standard test dose. The various phenols were added to the blood reservoir in volumes of 0.5 ml or 1 ml.

List of drugs used. Bradykinin (Synthetic Bradykinin, BRS 640, Sandoz AG). Adenosine triphosphate (ATP) (Adenosine 5-triphosphate disodium salt, crystalline from equine muscle, Sigma Chemical Company). Phenol (Phenolum, E. Merck AG). Tri-cresol (distilled by Nyegaard & Co, Oslo, Norway). Ortho-cresol (o-Kresol, Methylphenol A55137 Fluka AG). Para-cresol (p-Kresol, 4-Methylphenol A55141 Fluka AG). Meta-cresol (m-Kresol, 3-Methylphenol, A55139 Fluka AG). 2,3-Xylenol, 2,4-xylenol, 2,5-xylenol, 2,6-xylenol, 3,4-xylenol, 3,5-xylenol (all supplied by G. Stenerud, M. Sc. Institute of Chemistry, Department B, University of Oslo).

Fig. 1 shows the structural formulas of the 10 phenols tested.

Results

Injections of the standard test dose of ATP were carried out at 8 min intervals throughout the whole of the perfusion. The first few injections caused vasodilatation and the subsequent ones increasing vasoconstrictor responses. The effect of the various phenols was not tested until the vasoconstrictor response to ATP injections had become sufficiently marked, usually after 6 to 10 injections.



1 The structural formulas of the 10 phenols tested

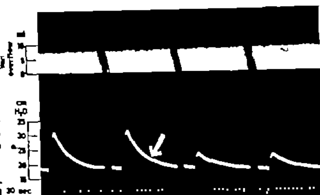


Fig. 2. Effect of injections of standard dose of ATP into the pulmonary arterial tubing before and after addition to the perfusate of p-cresol.
Isolated perfused lungs (IPL) rabbit, ♂, 3.0 kg. Upper tracing: ventilation overflow in ml. Lower tracing: pulmonary arterial pressure in cm H_2O. Blood flow (BF) through preparation 115 ml/min. Left atrial pressure (LAp) 2 cm H_2O. Four injections of ATP 50 µg were performed during the period illustrated. At arrow 1 mg of p-cresol was added to the blood reservoir.

O- m and p-cresol. The lower tracing in Fig. 2 is a recording of the pulmonary arterial pressure in a preparation during a period with four injections of ATP. Each injection was followed by an increase in pulmonary vascular resistance. The increase in resistance was calculated to be about 70 per cent following each of the first two injections. One mg of p-cresol was added to the blood reservoir at the arrow. The two subsequent ATP injections caused only a 25 per cent increase in vascular resistance. In Fig. 3 are plotted the results of a series of ATP-injections in a preparation. Each point represents the response to one injection of ATP. Doses of 1 mg of p-cresol were added to the perfusate three times during the period illustrated (at broken lines). Each time the ATP-induced increase in resistance was lowered to about half its previous value.

It was difficult to standardize the responses to injected ATP in the present preparation, partly because the response to a certain dose increased with time of perfusion (Fig. 3). Consequently comparisons of the effect of inhibitors against this response could only be semi-quantitative, and in the present investigation they have only been evaluated according to a rank scale.

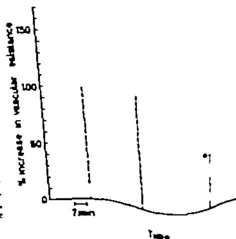


Fig. 3. Effect of p-cresol on ATP induced pulmonary vasoconstriction. A longer period of the experiment illustrated in Fig. 2 is shown. Each circle represents the response to one injection into the pulmonary arterial tubing of 50 µg of ATP measured as per cent increase in pulmonary vascular resistance in relation to the preinjection level. At broken lines, doses of 1 mg of p-cresol were added to the blood reservoir.

TABLE I Gas-chromatographic analysis of the commercial preparation of tri-cresol used in the present investigation

phenol	7,5 %
o-cresol	52,4 %
m-cresol	14 1 %
p-cresol	11,3 %
2,4- xylenol	7 7 %
2,5-xylenol	3,7 %
2,6-xylenol	1,6 %
non-identified	1 7 %

O-cresol was found to be about equally potent as an inhibitor towards ATP as was p-cresol. M-cresol was found to have only a moderate inhibitory effect.

Analysis of the commercial tri-cresol Tri-cresol is produced by fractionate distillation of coal-tare. The inhibitory effect of tri-cresol on the spontaneously developing vasoconstriction in isolated perfused lungs was originally discovered because tri-cresol is present as a preservative in some pharmaceutical preparations. The commercial tri-cresol preparation caused a marked inhibition of the ATP-response whereas an equal dose of either p- or o-cresol was found to be less potent. An analysis by gas chromatography showed that the tri-cresol contained about 20 per cent phenol and xylenols in addition to the cresols. The results of the analysis are listed in Table I

Phenol In the experiment of Fig. 4 phenol has been tested for ability to reduce the ATP induced vasoconstriction. Doses of 1 mg phenol were added to the blood reservoir at the broken lines and an equal amount of p-cresol at the dotted lines. Phenol had apparently no inhibitory effect on the ATP induced vasoconstriction.

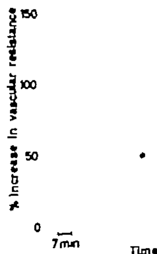


Fig. 4 Effect of phenol and of p-cresol on the ATP-induced pulmonary vasoconstriction. IPL, rabbit, ♂ 3.1 kg. BF 225 ml/min. LAP 1 cm H_2O. Doses of 1 mg of phenol were added to the blood reservoir at the broken lines and doses of 1 mg of p-cresol at the dotted lines. Abbreviations as in Fig. 2.

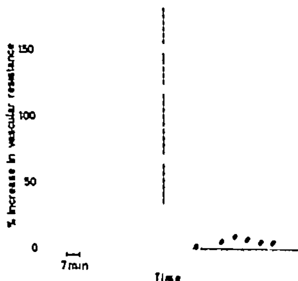


Fig. 5. Effect of 3,5-xylenol and 2,4-xylenol on the ATP-induced pulmonary vasoconstriction.
IPL, rabbit, ♀ 2.9 kg. BF 264 ml/min. LAp 1 cm H_2O. One mg of 3,5-xylenol was added to the blood reservoir at the broken lines, and 1 mg of 2,4-xylenol at the dotted line. Abbreviations as in Fig. 2.

Xylenols. There are six isomers of di-methyl phenol [illegible] shown in Fig. 1.

The 3,5-xylenol has both its methyl groups in [illegible] hydroxy group. This substance had no inhibitory effect on the [illegible] constriction.

The 2,4-xylenol has one methyl group in [illegible] and 2,6-xylenol has both its methyl-groups in [illegible] both a pronounced inhibitory effect on the AT[illegible]

Fig. 5 shows the results of one experiment where [illegible] mg of 3,5-xylenol was added to the blood reserv[illegible] 2,4-xylenol at the dotted line. The lack of effect [illegible] marked effect of the latter

Table II. The inhibitory effect on the ATP-induced [illegible] different phenols compared on [illegible] rank scale

Substance	[illegible]
phenol	[illegible]
o-cresol	[illegible]
m-cresol	
p-cresol	[illegible]
2,3-xylenol	
2,4-xylenol	
2,5-xylenol	
2,6-xylenol	
3,4-xylenol	
3,5-xylenol	0

2,3-xylenol 2,5-xylenol and 3,4-xylenol all have one methyl-group in m-position and one methyl-group in either o- or p-position in relation to the hydroxy group. These substances were equally potent. They were however not as efficient as 2,4- and 2,6-xylenol.

The inhibitory effects of the ten phenols are compared on a rank scale in Table II.

An arterial injection of 10 μg bradykinin towards the end of an experiment, always caused a marked vasoconstriction, even when the inhibition of the ATP induced vasoconstriction was nearly total. Thus, the inhibitory effect of the different phenols towards ATP-induced vasocontriction is not due to an unspecific paralysis of vascular smooth muscle.

When the response to a certain dose of ATP was completely abolished by a large dose of a phenol inhibitor it was also always possible to get the vasoconstrictor response back by increasing the dose of ATP.

Discussion

The pressor response to ATP in the pulmonary vascular bed is seen also in vivo (Emmelin and Feldberg 1948, Reeves et al. 1967) and it might deserve more attention as far as the underlying mechanism is concerned.

Reeves et al. (1967) suggest that the pressor effect of injected ADP and also of ATP in the pulmonary vascular bed of unanesthetized calves is caused by aggregation of thrombocytes and by a subsequent vascular obstruction. In our preparation injected ATP caused a marked pressor effect even when the lungs were perfused with plasma without thrombocytes (Hauge et al. 1966 a). If the smooth muscle was paralyzed with papaverin, injected ATP did not cause any change in vascular resistance. These findings suggest that the greater part of the increase in vascular resistance was caused by vasoconstriction and not by thrombocyt aggregation.

The interference of phenols with this pulmonary vasoconstrictor response to ATP is an interesting effect, which so far no other drugs have been found to exert. Not only are the most effective phenols very potent in their ATP-counteracting ability in the present preparation, but there seems to be an interesting relationship between structure and inhibitory potency. This relationship might on further analyses reveal information as to be the nature of the interference with the ATP effect, and possibly also as to the nature of the ATP effect itself.

In a preparation of the present type there are considerable difficulties in measuring drug responses and inhibitory effects. Accurate comparisons of various inhibitors would require a less complex preparation, preferably one consisting of an isolated piece of muscle, where many tests could be performed in a reasonable time. In spite of these difficulties a definite pattern has been revealed in the results. A methyl group in either o- or p-position in relation to the hydroxy group increased the inhibitory effect of the phenol, whereas a methyl group in m-position resulted in little inhibitory effect.

The way in which the phenols interfere with the responses to added ATP is unknown. There seems to be three possible mechanisms.

The phenols may react with ATP in the blood. No chemical reaction of this type is known. Further Jonsson (1966) has shown that m-cresol does not inhibit the vasodilatator effect of ATP in the intestinal vascular bed in the cat. Thus, a chemical reaction between phenols and ATP is not a very probable explanation for the inhibitory effect.

Another possibility is that the inactivation of ATP by ATP-ase is accelerated either in the blood or in the preparation tissue. Aldridge and Stoner (1960) thus found an ATP-ase potentiating effect of 3,5-dinitro-o-cresol in liver mitochondria. Born (1954) found that a 10^{-4} M concentration of 2,4-dinitrophenol in the perfusate brought about a gradual decrease in perfusion pressure in isolated perfused rabbit lungs. Simultaneously ATP disappeared from the lung tissues.

The third possibility is that the effect of ATP on the smooth muscle is directly inhibited on a receptor level by the phenols. This last explanation is perhaps the most likely one.

We want to thank R. Sorlie for skilful technical assistance, G. Stenerud, Institute of Chemistry Department B, University of Oslo for supplying us with all the xylenol isomers, and K. Oydvin, Norsk Medisinaldepot, for performing the gaschromatographic analyses. P. K. M. Lunde has been a Research Fellow of the Norwegian Research Council for Science and the Humanities. Financial support from this Council and from the Norwegian Council on Cardiovascular Diseases is gratefully acknowledged.

References

ALDRIDGE, W. N. and H. B. STONER, The behaviour of liver mitochondria isolated from rats with different body temperature after limb ischaemia or after injection of 3, 5-Dinitro-o-cresol. *Biochem. J.* 1960. *74* 148—154.

BORN, G. V. R., Acute oedema in isolated, perfused lungs of rabbits. *J. Physiol.* (Lond.) 1954. *124* 502—514.

EMMELIN, N. and W. FELDBERG, Systemic effects of adenosine triphosphate. *Brit. J. Pharmacol.* 1948. *3* 273—284.

HAUGE, A., P. K. M. LUNDE and B. A. WAALER, Vasoconstriction in isolated blood-perfused rabbit lungs and its inhibition by cresols. *Acta physiol. scand.* 1966 a. *66* 226—240.

HAUGE, A., P. K. M. LUNDE, B. A. WAALER and L. WALLOE, The inhibitory effect of various phenols upon ATP-induced vasoconstriction in isolated perfused lungs. *Acta physiol. scand.* 1966 b. *68* Suppl. 277. 70.

JONSSON, O. Personal communication. 1966.

KONZETT, H. and R. ROSSLER, Versuchsanordnung zu Untersuchungen an der Bronchialmuskulatur. *Naunyn-Schmiedeberg Arch. exp. Path. Pharmak.* 1940. *195* 1—7.

REEVES, J. T., P. JOKL, J. MERIDA and J. E. LEATHERS, Pulmonary vascular obstruction following administration of high-energy nucleotides. *J. appl. Physiol.* 1967. *22(3)* 475—479.

Acta physiol. scand. 1968. 72. 338—349

Medical Research Council Department of Clinical Research, University College Hospital Medical School, London, England

Transmembrane Electrical Potential Differences and Ionic Composition of Mucosal Cells of Rat Colon

by

C. J Edmonds and O E. Nielsen

Received 7 July 1967

Abstract

Edmonds, C. J and O E. Nielsen. *Transmembrane electrical potential differences and ionic composition of mucosal cells of rat colon.* Acta physiol. scand. 1968. 72 338—349

Using glass microelectrodes, stable electrical potential differences (p d) have been measured across the cell membranes of mucosal cells of rat colon in vivo and in vitro in tissue mounted in chambers. The intracellular content of Na, K, Cl and water has been determined under similar conditions.

The serosal transmembrane p.d. was slightly greater than the luminal transmembrane p d, in both cases the inside of the cell being negative with respect to the exterior. The cell Na content was low and cell K high when compared with extracellular fluid but the transmembrane p d. measurements were considerably less than expected if they were simply K diffusion potentials.

The present results have been considered in their relationship to the known ionic fluxes and it is suggested that a) Na and K 'pumps' are present on the serosal side of the mucosal cells and b) that, since the transmembrane fluxes of Na and Cl are inconsistent with the observed transmembrane potentials and the mucosal ionic composition, the bulk of these ions cross the mucosa either through special cells or by pathways between the cells.

In common with a number of ion transporting epithelia, the colonic mucosa has been observed to be electrically polarized (Cooperstein and Hogben 1959 Curran and Schwartz 1960) The transmucosal potential difference (p d) is relatively small under normal circumstances, rarely exceeding 20 mV in the rat colon with the serosal surface charged positively with respect to the lumen. Considerable fluxes of Na, K and Cl ions take place across the mucosa (Curran and Schwartz 1960 Edmonds 1967 b, c) These ions have to pass through at least two membranes of the epithelial cells, one on the luminal and one on the serosal side, and it would be of value to know the p.d. across each cell membrane in addition to the overall transmucosal p.d. Then, knowing the ionic composition of the cells, more detailed

analysis of the ionic movements could be attempted, in a way for example, that has been done for the proximal renal tubule (Whittembury 1960, Giebisch 1961).

In the present paper the results of measurements of electrical p.d. across each face of rat colonic epithelial cells are presented, together with measurements of the intracellular ionic composition of colonic mucosa. The results are analysed in relationship to the known ionic movements.

Transmucosal p.d. is the p.d. measured between electrodes placed on either side of the colon mucosa. This has the same value as the p.d. between an electrode in the gut lumen and one in the peritoneal cavity making electrical contact through isotonic saline with the peritoneal surface of the gut (Edmonds 1967a).

Transmembrane p.d. is the p.d. measured between an electrode within the cell and one outside it. This is prefixed by luminal or serosal according to the side on which the extracellular electrode is placed.

Methods

Male albino rats weighing 300—350 g were used. For 7—10 days before experiment they were fed boiled rice in proportions of 100 g dry rice to 200 ml distilled water with addition of 3 m-moles of KCl per 100 g dry rice. They were given 0.5 % NaCl solution as drinking fluid.

Operative procedure. The rats were anaesthetized with intraperitoneal pentobarbitone 6 mg/100 g body weight, and the abdominal cavity opened by a midline incision. Segments of ascending and descending colon each about 3 cm long, were prepared, the bowel being opened in two places using cutting cautery and the lumen rinsed out with warm 150 mM saline. For the in vitro experiments, the colonic segments were removed on a glass tube (ext. diam 0.7 cm) and the muscular layer quickly stripped from the underlying mucosa, a procedure easily accomplished as the tissue cleaved readily between the muscular coat and the muscularis mucosa. The tube of mucosal epithelium was then cut longitudinally, opened as a sheet and mounted in a chamber the details of which will be described later. For the in vivo experiments, a glass tube (ext. diam. 0.6 cm) was passed through the lumen, one end of the tube being rigidly fixed in a holder thus eliminating disturbing respiratory movements. An incision of about 0.5 cm long was made through the muscular coat of the colon exposing the underlying mucosa. The microelectrode was introduced where the mucosa was exposed.

Fig. 1 shows the tissue and microelectrode arrangement in the in vitro experiments. The chamber was constructed from a perspex cylinder of 2.5 cm ext. diam. The lower part contained a central well of diam. 1.5 cm and depth 1.5 cm and had holes in the wall to provide passage for the electrodes. The upper part was made from the same material but drilled so as

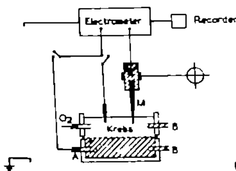


Fig. 1. Diagram of chamber and microelectrode arrangement. M, microelectrode filled with 3 M KCl. A, reference electrode; B, electrodes for measuring transmucosal p.d. Both A and B contained 150 mM NaCl 4% agar and were connected to the electrometer through Ag—AgCl junctions.

to form a hollow cylinder of length 1 cm. It also had holes in the wall to allow access to an electrode and to an O_2 supply tube; it fitted exactly onto the lower part being secured in position by three screws. The colon mucosa was mounted on the lower part with its serosal side uppermost. The upper part of the chamber was placed in position and fixed by the screws so that the tissue was firmly clamped between the two parts of the chamber. The serosal side was bathed by Krebs-Ringer bicarbonate solution (referred to subsequently as Krebs solution) of composition NaCl 118 mM, $NaHCO_3$ 24 mM, KCl 4.8 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, glucose 14 mM and was gassed continuously by a mixture of 95 % O_2–5 % CO_2. The transmucosal p.d. was measured at intervals by 150 mM NaCl agar bridges connected through calomel electrodes to the input of the electrometer. In advancing the electrode through the bath to the surface of the tissue the microscope was used to observing the tip. The tissue was entered by advancing the electrode very slowly to avoid breakage of the tip. Immediately the tip penetrated the tissue surface the electrometer recorded small and quickly varying potentials and further advance of the electrode was made without the use of the microscope. In preliminary experiments the lower part of the chamber was filled with Krebs solution but tissue flaccidity made impalements difficult and very often the electrode tip broke. By filling the lower part with agar 150 mM NaCl upon which the tissue rested, this effect was eliminated. With this technique if dimpling occurred on attempting to insert the electrode only small and unstable p.d. measurements could be obtained, probably indicating a blunt electrode tip (Bülbring and Hooton, 1954).

In the in vitro technique two indifferent electrodes were present, one in the upper and another in the lower part of the chamber. In a number of experiments in which the tissue possessed a sufficient transmucosal p.d. the transmembrane p.d. was measured across each face of the cell, either the serosal or the luminal side electrode acting as the indifferent electrode. At the beginning of these experiments it was always ensured that the two indifferent electrodes had an asymmetry p.d. not greater than 0.5 mV.

In the in vivo experiments the technique of insertion of the microelectrode was similar. The indifferent electrode was placed in the abdominal cavity, electrical contact being ensured by the introduction of a little Krebs solution.

Electrical p.d. measurement. Microelectrodes were drawn from Pyrex tubing of approximately 2 mm ext. diam. using an Alexander and Nastuk type puller. The electrodes were filled with filtered 3 M KCl by gently boiling for about 15 min at reduced pressure. The resistance and tip potential of each electrode were measured in Krebs solution, electrodes with tip potentials greater than 5 mV or with resistances greater than 6 MΩ being rejected (Adrian, 1956). The microelectrode was held firmly but not rigidly in a perspex tube containing agar 150 mM NaCl and containing an Ag–AgCl junction connecting by means of a short screened lead to the input of a Vibron electrometer (E.I.L. model 33B-2). The tube was mounted in a Prior micromanipulator and impalements of tissue were carried out using a stereoscopic binocular microscope at × 40 magnification. The indifferent lead was formed by a glass tube of 0.3 cm ext. diam. filled with agar 150 mM NaCl solution into which a chlorided silver wire extended. Equipment for measuring tip potentials and resistances of the microelectrodes was mounted in the apparatus so that the condition of electrodes could be checked before and after impalements. The electrometer had a grid current less than 10^{-13} A and an input impedance of 10^{14} Ω. All equipment was contained within a Faraday cage to which the electrometer was earthed. The electrometer output was fed to a pen recorder and p.d. measurements monitored throughout the experiment.

Estimation of extracellular water, Na, K and Cl. The estimations were carried out on tissues which had been exposed in vitro in Krebs solution for 90 minutes and on tissues freshly removed from rats.

(i) *In vitro method.* The colonic mucosal layer was obtained by stripping off the muscular coat as described above. It was then mounted in a small chamber basically similar to that described by Ussing (1949). Krebs solution was placed on both sides of the mucosa and in addition inulin-^{14}C[1] was placed on the serosal side at an activity of about 0.5 μCi per ml. In some preliminary experiments inulin-^{14}C was placed instead on the mucosal side but as there was no significant uptake of radioactivity by the tissue these will not be considered. The solutions on both sides of the tissue were gassed with 95 % O_2–5 % CO_2 and allowed to remain at room temperature (23° C) for 90 min. The exposed tissue was then removed from the chamber, blotted several times using Whatman ashless filter paper and rapidly weighed giving the

[1] Inulin carboxyl-^{14}C was obtained from New England Nuclear Corp., Mass. U.S.A. To ensure that it behaved like chemical inulin, a mixture of inulin-^{14}C and inulin (BDH) was injected into a rat whose renal pedicles had been tied. After 3 hrs the ratio of inulin-^{14}C to chemical inulin in the blood was determined. This was found to be not significantly different from the ratio of the injected solution.

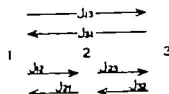


Fig. 2. Ionic flux rates in three compartment system. The flux rate of stable ion is represented by J with subscripts to indicate the direction of flow

tissue wet weight. The tissue water content was determined by drying in an oven at 98 C until its weight became constant, this being achieved in about 18 hrs, to give the tissue dry weight. The tissue was then transferred to silica tube, 0.5 ml of 0.01 N HNO_3 added and the tube shaken for 8 hours. Preliminary experiments showed that by this time the extraction of Na, K and Cl and inulin ^{14}C was complete, and their quantity could be estimated in the extraction fluid. Initial trial experiments showed that the procedure involved no significant loss of Na, K, Cl or inulin C.

(b) *In vivo method.* The abdomen was opened initially and both renal pedicles ligated to avoid the considerable renal loss of inulin which would otherwise have occurred. An intravenous injection of 0.5 ml of 150 mM saline containing about 1 μCi of inulin C was then made into the external jugular vein. After injection the rats were allowed to recover from the anesthetic and left for 3 hrs to allow the inulin to distribute itself throughout the extracellular fluid (Ledingham 1953). At the end of this time, the abdomen was re-opened, the gut contents washed out with warm isotonic saline and pieces of ascending and descending colon, each about 3 cm long removed on glass tubes. The muscle coat was stripped off and the mucosa opened out as sheet. The water content was determined by drying and the tissue extracted in 0.01 N HNO_3 as already described. Blood was taken from the internal carotid artery into heparinised tubes for electrolyte and inulin ^{14}C measurements.

Chemical and isotope methods. Na and K were determined by an EEL flame photometer to an accuracy of not less than ±1.5 % (this and all subsequent accuracies are stated as ±1 S.D. based on 10 replicates). Cl was determined by potentiometric titration (accuracy ±1.8 %). Inulin ^{14}C content of the extract and of plasma was measured by pipetting 0.2 ml on to Al planchets followed by oven drying at 98 C and the counting with an end window G.M. counter (20th Century Electronics EW 2C) to an accuracy of ±3 %.

Calculations. The simplest model of the mucosal epithelium in which the cell layer is regarded as homogenous single compartment separating the luminal fluid from the extracellular fluid is shown in Fig. 2. The ions crossing the mucosa are considered in this model to mix completely with the ions of the mucosal layer. In examining the relationships between the ionic fluxes, the following symbols will be used

J = flux rate of Na (or Cl) from compartment i to compartment j (for example, in μmoles/per min)

J = net flux rate of Na (or Cl)

In the steady state when net transfer of Na and Cl from lumen to ... is taking place then

J — J = J — J = J — J = J

hence

$$\frac{J_{13}}{J} = 1 + \frac{J}{J} \quad — (1)$$

and

$$\frac{J}{J_{31}} = 1 - \frac{J}{J_{31}} \quad (2)$$

Thus from the flux ratio ... each face and J_n, the individual flux rates can be calculated. Flux rates will be expressed as mmole per min per cm of gut length.

Results

P.d. measurements

The criterion for an intracellular localisation of the electrode tip was a sudden marked increase in negativity of the recording electrode stable for five or more

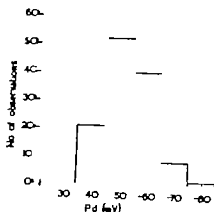


Fig. 3. Summary of results of transmembrane potential measurements in single cells of rat colon mucosa with the indifferent electrode on the serosal side.

minutes. Very often an increase in the measured potential of a few millivolts occurred immediately after penetrating a cell. A similar effect has been observed in impalements of nephron cells (Giebisch 1958) and may have been due to progressive sealing around the lectrode tip. The measured potentials then remained stable for up to 10—15 min and then a slow decline usually occurred. In several instances, on advancing the electrode after this decline it was observed that a new recording of the original magnitude was often made, presumably indicating that another mucosal ll had been entered.

In vitro micropunctures satisfying the above criteria were done 57 times in cending colon and 38 times in descending colon. The average p.d. measured between the intracellular and th serosal side electrodes was 54 mV in the case of the ascending colon and 53 mV for the descending. The mean p.d. obtained from the total 95 observations was 54±18 mV (± 2 s.d.) the inside of the cell being negative with respect to the exterior

In ivo measurements showed an average potential of 51 mV in 22 ascending colon recordings and 54 mV in 6 recordings in the descending colon, the p.d. again being measured with the recording electrode placed intracellularly and the indifferent electrode on the serosal side. The mean p.d. in the total 28 observations as 52±14 mV (±2 d. The difference between the results of the in ivo and in itro experiments in ascending and descending preparations was not statistically significant. In fig. 3 are summarized data on the frequency distribution of 123 colon transmembrane potentials.

The average transmucosal p.d. of the colon preparations was 7 mV (range 4 to 14 mV) the mucosal side being negative with respect to the serosal. These values are similar to those previously observed in rat colon (Edmonds 1967b) In the in vitro preparation, the transmucosal p.d. gradually declined over the course of 1 to 9 hrs but the transmembrane potentials changed little during this time.

TABLE I. Transmucosal p.d. compared with serosal side and luminal side transmembrane p.d. in eight in vitro experiments

Exp. no.	Transmembrane p.d. Serosal mV	Luminal mV	Difference mV	Transmucosal p.d. mV
1	48	37	11	10
2	59	52	7	7
3	58	49	9	8
4	55	45	10	10
5	51	44	7	6
6	58	49	9	8
7	58	46	12	11
8	50	36	14	14
Mean ± s.d.	54.6 ± 4.7	44.7 ± 5.7	9.9 ± 2.4	9.3 ± 2.6

TABLE II. Tissue weight, water content and intracellular fluid (i.c.f.) Na, K and Cl content of mucosal epithelium from rat colon

	Dry wt (mg)	Water % of wet wt	I.c.f. % of total water	I.c.f. composition Na	K	Cl	Number of specimens
				mmoles/kg cell		water	
Fresh	4.1	76.2	68	73	158	—	12
s.d.	1.9	3.3	10	9.6	15		
In vitro	42.9	75.5	60	57	192	45	12
s.d.	7.6	1.9	7.4	12	18	14	

In some experiments, the p.d. between the intracellular and serosal side electrode was compared with that between the intracellular and mucosal side electrode (Table I). The serosal side p.d. was always greater than that of the mucosal side and the sum of the two, assuming they were in series, was in excellent agreement with the transmucosal p.d.

Tissue electrolyte and water content

The water content of the in vitro specimens was slightly greater than that of the fresh colon mucosa but the difference was not significant. In both groups the intracellular fluid (i.c.f.) formed a similar proportion of the total tissue water (Table II). The pieces of tissue which came from the chambers were very much smaller than those freshly removed from rats. The Cl content was therefore low so that with

TABLE III Na, K and Cl content of rat plasma, extracellular fluid (e.c.f.) and Krebs-Ringer bicarbonate solution

	Plasma mM	e.c.f. mM	Krebs-Ringer bicarbonate mM
Na	144	144	145
s.d.	2.8		
K	4.6	4.6	5.5
s.d.	0.4		
Cl	108	117	125
s.d.	3.9		

The plasma data were obtained from 9 rats. The e.c.f. values have been calculated on the basis of water content of 0.94 ml per ml of plasma and Donnan factors of 0.94 for Na and 1.02 for Cl (Mannery 1954)

our method satisfactory Cl determinations could not be made on the in vitro specimens. The amount of Cl in the cells of fresh colon mucosa was fairly high although when expressed in relationship to the amount of water present in the mucosal layer Cl concentration was less than half the extracellular fluid (e.c.f.) concentration (Table III). Both in the in vitro and in the fresh specimens, the amount of Na in the mucosal layer was substantially less than K but the difference was greater in the ... specimens. Expressed in relationship to the water content of the mucosa, the ... specimens had a Na content significantly less than that of the in vitro ($P<$ .05) whilst having a K content greater than the in vitro tissues ($P<0.005$)

Ionic fluxes at the luminal and serosal cell membrane

In the simple model of Fig. 2, the mucosal epithelium is represented as a single compartment with boundaries formed by the luminal and serosal side membranes of the cells. Similar models have been used in discussion of a variety of other epithelia, for example frog skin (Koefoed-Johnsen and Ussing 1958), frog stomach (Harris and Edelman 1964), toad bladder (Leaf, Andersen and Page 1958), renal tubular epithelium (Giebisch 1961). For such a model, by using the data obtained in the present experiments from the ionic composition of the mucosal cells and the transmembrane p.d.s., it is possible to calculate the ratio of the inward and outward fluxes of each ion crossing the cell membrane. This calculated flux ratio however is only applicable to situations where the ionic movements are due to electrochemical gradients only and where there is no interaction with other ions or molecules. Then the flux ratio equation (Ussing 1949) can be applied which can be written for the luminal membrane as

$$\frac{J}{J_{11}} = \frac{\gamma\ [C]}{\gamma\ [C]_{1}} \quad \exp\left(\frac{\Delta VF}{RT}\right)$$

TABLE IV Comparison of the calculated and observed K flux ratios

	Calculated	Observed
Luminal side J/J	0.13	0.14
Serosal side J/J	0.18	1.2

The calculated ratios were calculated by the flux ratio equation and from the results obtained in the present investigation.

TABLE V Estimated ionic flux rates and ratios of Na and Cl

Ionic flux rates (mμmoles/min/cm) and flux ratios

		Na	Cl
Luminal side	J	263	—
	J	13	—
I	J/J	20.2	0.64
II	J/J	> 1.0	> 1.0
Serosal side	J	—	295
	J	—	625
I	J/J	27.5	0.47
II	J/J	< 1.0	< 1.0

The flux ratios I were calculated by the flux ratio equation and from the results obtained in the present investigation. The flux ratios II were based on the results of transmucosal ionic flux measurements (Fig. 4) and thus can only be stated as greater or less than 1.0. The ionic flux rates were calculated from flux ratios I and the net flux rates using equations (1) and (2). The serosal side Na and the luminal side Cl flux rates could not be calculated in this way since flux ratio I indicated net flow in the opposite direction to that observed in transmucosal flux measurements.

where γ = activity coefficient, C = ionic concentration, ΔV is the p.d. across the membrane, and R, T and F have their usual significance. The subscripts are as shown in Fig. 2 and it was assumed that the activity coefficients were the same for ions inside and outside the cells. The assumptions of the flux ratio equation have recently been reviewed (Ussing 1965) and will not be further considered here except to note that we have assumed that the intracellular ions behave as though they were in solution in the cell water and further that the mucosal cell layer behaves as an homogenous layer in which ions mix completely as they cross the mucosa. The flux ratios calculated from the present observations are given in Tables IV and V. It remains to compare these results with those obtained in other experiments in which ionic fluxes have been measured using radioisotope methods.

In the case of K, direct comparison between the flux ratios calculated above with those found in radioisotope studies is possible since the latter have been recently measured (Edmonds 1967d). Fig. 4 summarizes the mean values of the K fluxes [illegible]

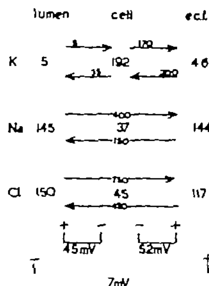


Fig. 4 Ionic composition, unidirectional flux rates and electrical p. d. measurements summarized for the in condition of rat colon mucosa. The numbers attached to the arrows refer to the unidirectional fluxes expressed in mμmoles/min/cm gut length. The other numbers refer to the ionic composition of plasma and luminal fluid expressed as mM and the intracellular composition expressed as moles/kg cell water. In the case of K the flux rates across the cell surfaces are given. In the case of Na and Cl only the transmucosal fluxes are known and the values for Na are those obtained after the exchange diffusion component has been subtracted (Curran and Schwartz 1960)

each side of the mucosa and in Table IV the flux ratios are compared. On the luminal side there is good agreement between the flux ratios determined either way. On the serosal side however the value of J_{23}/J_{32} obtained from radioisotope measurement is considerably greater than expected from the observed p.d. and ionic concentrations.

Na and Cl In the case of Na and Cl the actual fluxes across each face of the mucosal layer have not been directly measured but the transmucosal fluxes have been determined (Curran and Schwartz 1960, Edmonds 1967b) and the mean values are shown in Fig. 4. The net flux J is also known being simply given by the difference between J and J_3. Thus by taking the flux ratios calculated from application of the flux ratio equation to the data of the present experiments together with the values of J then using equations (1) and (2) it should be possible to calculate the flux ratios at each side of the mucosa. The results of this procedure are shown in Table V. With Na, this method could not be applied to the serosal side since on inspection there is an obvious contradiction in that the calculated flux ratio J_{23}/J_{32} was 27.5 and yet there must have been a net flow of Na from cell layer to e.c.f., that is $J_{23} > J_{32}$. A similar contradiction is present for the luminal side in the case of Cl. Here again as Cl was being absorbed $J > J_{21}$ yet the value of J_{12}/J_{21} calculated using the flux ratio equation indicated that $J_{12} < J_{21}$ (Table V).

Comparison of the face fluxes with the transmucosal fluxes (Table V and Fig. 4) shows a further discrepancy. The transmucosal flux in one direction cannot exceed any individual face flux in the same direction (Leaf 1958). For example, both J_{12} and J_{23} must exceed J_{13}. Thus it would be concluded from Table V that in the case of Na, J must be less than 263 mμmoles/min/cm and J_{31} less than 13 mμmoles/min/cm. Yet the measured transmucosal fluxes were much greater than these values

(Fig 4) For Cl, Table V indicates that J_{12} must be less than 625 mμmoles/min/cm and J_{21} less than 295 mμmoles/min/cm. Again both figures are less than those observed from radioisotope flux measurements (Fig 4) although the difference is relatively small and may be due to experimental variations.

Discussion

Our investigations show that the mucosal cell of rat colon is electrically asymmetrical, the luminal side having a transmembrane p.d. less than that of the serosal side, the difference between the two being equal to the transmucosal p.d. This situation is strikingly similar to that observed in the proximal renal tubular cell of Necturus (Giebisch 1958, Whittembury and Windhager 1961) Both renal and colon mucosal cells are involved in absorptive and secretory processes and the electrical asymmetry is probably a result of these activities (Giebisch 1961)

A high K content is a feature of most cells and in several cell types which have been extensively studied e.g. muscle cells, the transmembrane p.d. behaves as though it were a K diffusion potential associated with the considerable K gradient across the cell membrane (Kernan and Conway 1955 Adrian 1956, Vaughan Williams 1959 Kernan 1963 Kernan 1965) In the renal tubular cell of Necturus, Giebisch (1958) measured an average transmembrane p.d. of 72 mV but using the Nernst equation he calculated the expected p.d. as 94 mV assuming it to be simply a K diffusion potential. In our in vitro experiments the average transmembrane p.d. was 54 mV while that expected from the Nernst equation was 87 mV The corresponding observed and expected values for the in vivo experiments were 52 mV and 107 mV respectively Thus it appears that a K diffusion p.d. alone cannot explain the transmembrane p.d. of colonic mucosal cells, a finding not altogether unexpected as a considerable flow of other ions takes place across the tissue so that pure K electrode behaviour at either the luminal or serosal cell membranes would seem unlikely (Hodgkin and Katz 1949)

In electrical polarization, in the flows of water Na, K and Cl ions, and in the active transport of Na there exist notable similarities between the colonic and renal tubular epithelium. It is pertinent therefore to consider whether a model such as that proposed to account for the observations on renal tubular cells (e.g. Giebisch 1961) is relevant to colonic mucosa. In this model there are basically three compartments as in Fig 2 and the transport of ions across the epithelium involves complete mixing with the ions within the epithelial cells On one face of the cell layer the movement of ions takes place under the influence of the electrochemical gradient only On the other face in addition to this movement, active transport of Na and K takes place tending to extrude Na from the cells and transfer K into the cell

Calculation of the passive ionic flux rates expected from the present observations showed several discrepancies indicating that passive diffusion of ions along electrochemical gradients did not adequately account for the ionic fluxes The discrepancy in the Na and K flux ratios on the serosal side can be explained by postulating

active transport of these ions at this surface, Na being 'pumped' out of the cell into the e.c.f. and K in the reverse direction. Such a model is essentially like that proposed for renal tubular cells and toad bladder epithelium. However it was found that on the luminal side also, the Na and Cl movements did not appear to be consistent with passive diffusion of the ions along electrochemical gradients. A solvent drag effect due to net water transport from lumen to plasma could be responsible for the anomaly in the case of Cl although it seems unlikely that the effect would be great enough to convert the expected flux ratio J_{13}/J_{31} from 0.64 to a value >1.0. Regarding the Na flux ratios of the luminal side, Solomon (1963) in analyzing the face fluxes of Necturus renal tubule observed a similar difficulty in that the flux J_{21} was considerably greater than was expected from a passive flow due to the electrochemical gradient. He suggested the possibility that Na may have been actively transferred from cell to lumen. Plainly however this suggestion would not be adequate for the present situation since the values of both J_{13} and J_{31} as calculated from the flux ratio equation and Jn are far too low to be compatible with the known transmucosal fluxes. Exchange diffusion of Na (Ussing 1949) across the luminal membrane if adequate in amount could account for an anomaly of this type. However in the data given in Fig 4 the transmucosal Na fluxes have already been corrected to remove the exchange diffusion component. Further Curran and Schwartz (1960) found that the Na flux J had an average value of about 200 $\mu\mu$moles/min cm even when the lumen was Na free when exchange diffusion could not be occurring. Exchange diffusion is thus insufficient to explain the discrepancies.

A possible explanation of these difficulties is that the intermediate compartment is not homogenous as is supposed in the three compartment model. It is possible that a considerable proportion of Na and Cl movement takes place through a small number of special cells with electrical properties and ionic composition not apparent in the study of the mucosa as a whole or alternatively much of the Na and Cl crossing the mucosa may pass between rather than through the cells so that in fact the ions do not traverse the cell membranes across which exist the relatively high potentials observed in our experiments. The mechanisms for active Na transport across the epithelium could lie in association with these special cells or pathways. The evidence at present leaves the problem in doubt but it is of interest to note that intracellular shunts have been suggested in frog skin to account for some of the phenomena of Na transport (Ussing 1963) and there is also some electron microscopy evidence for the existence of channels between the cells of Na and Cl transporting epithelia (Tormey and Diamond 1966).

One of us (O.E.N.) was in receipt of a grant from the Danish Carlsberg Foundation. We thank Dr E. E. Pochin for helpful discussion during the preparation of the manuscript.

References

ADRIAN, R. H., The effect of internal and external potassium concentration on the membrane potential of frog muscle. *J. Physiol.* (Lond.) 1956 *133* 631—658.
ALEXANDER, J. T. and W. L. NASTUK, An instrument for the production of microelectrodes used in electrophysiological studies. *Rev. sci. Instrum.* 1953 *24* 528—531.

BÜLBRING, E. and I. N. HOOTON, Membrane potentials of smooth muscle fibres in the rabbits sphincter pupillae. *J. Physiol.* (Lond.) 1954 *125* 295—301

COOPERSTEIN, I. L. and C. A. M. HOGBEN, Ionic transfer across the isolated frog large intestine. *J. gen. Physiol.* 1959 *42* 461—473.

CURRAN P F and G F SCHWARTZ, Sodium, chloride and water transport by rat colon. *J. gen. Physiol.* 1960 *43* 555—571

EDMONDS, C. J The gradient of electrical potential difference and of sodium and potassium along the caecum and colon of normal and sodium depleted rats. *J Physiol.* (Lond.) 1967a. In press

EDMONDS, C. J Transport of sodium and secretion of potassium and bicarbonate by the colon of normal and sodium depleted rats. *J. Physiol.* (Lond.) 1967b. In press.

EDMONDS, C. J Transport of potassium by the colon of normal and sodium depleted rats. *J Physiol.* (Lond.) 1967c. In press.

EDMONDS, C. J (1967d) In prep.

GIEBISCH, G Electrical potential measurement in single nephrons of Necturus. *J. cell. comp. Physiol.* 1958 *51* 221—239

GIEBISCH, G., Measurements of electrical potential differences on single nephrons of the perfused Necturus kidney *J. gen. Physiol.* 1961 *44* 659—678.

HARRIS, J B. and I S. EDELMAN, Chemical concentration gradients and electrical properties of gastric mucosa. *Amer J Physiol.* 1964 *206* 769—782.

HODGKIN, A L. and B. KATZ, The effect of sodium ions on the electrical activity of the giant axon of the squid. *J Physiol.* (Lond.) 1949 *108* 37—77

KERNAN, R. P *C U A* London (Butterworths) 1965 p. 71

KERNAN, R. P The resting potential of isolated rat muscles measured in plasma. *Nature* (Lond.) 1963. *200* 474—475.

KERNAN, R. P and E. J CONWAY Muscle fibre membrane potentials in relation to external potassium concentration. *Abst. Int. Biochem. Congr.* Brussels 1955.

KOEFOED-JOHNSEN, V and H. H. USSING, The nature of the frog skin potential. *Acta physiol. scand.* 1958. *42*. 298—308.

LEAF A Measurement of the permeability of the two surfaces of living membrane. *Science* 1958. *128* 144—145.

LEAF A., J ANDERSON and L. B. PAGE, Active sodium transport by the isolated toad bladder *J. gen. Physiol.* 1958. *41* 657—668.

LEDINGHAM, J. M., The distribution of water sodium and potassium in heart and skeletal muscle in experimental renal hypertension in rats. *Clin. Sci.* 1953 *12* 337—349

MANERY J F Water and electrolyte metabolism. *Physiol. Rev.* 1954. *34* 334—417

SOLOMON, A K., Single proximal tubules of Necturus kidney VII. ion fluxes across individual faces of the cell. *Amer J Physiol.* 1963 *204* 381—386.

SOLOMON, S Transtubular potential differences of rat kidney *J. cell. comp. Physiol.* 1957 *49* 351—365.

TORMEY J M. and M. J DIAMOND, An ultrastructural model for isotonic fluid transport in epithelia *Gastroenterology* 1966. *51* 582—593

USSING, H. H The distinction by means of tracers between active transport and diffusion *Acta physiol. scand.* 1949 *19* 43—56.

USSING, H. H Transport of electrolytes and water across epithelia. *Harvey Lect* 1963—64 London Academic Press Inc. Ltd.

VAUGHAN WILLIAMS, E. M., The effect of changes in extracellular potassium concentration on the intracellular potentials of isolated rabbit atria *J Physiol.* (Lond) 1959 *146* 411—428

WHITTEMBURY G Ion and water transport in the proximal tubule of the kidney of Necturus maculosus *J. gen. Physiol.* 1960. *43* 43—56.

WHITTEMBURY, G. and E. E WINDHAGER, Electrical potential difference measurements in perfused single proximal tubules of Necturus kidney *J. gen. Physiol.* 1961 *44* 679—68

Acta physiol. scand. 1968, 72, 350—365

From the Institute of Neurophysiology University of Copenhagen, Denmark

Spontaneous Afferent Discharges and Spontaneous Intrafusal Contractions in Isolated Muscle Spindles of the Frog

By

SAMIA AL AZHARIA JAHN

Received 7 July 1967

Abstract

JAHN, S. A. A. *Spontaneous afferent discha ge and spontaneou int afusal contractions n isolated mu cle spindles of the f og* Acta physiol. scand. 1968. 72. 350—365

Intrafusal recordings from isolated muscle spindles of the frog showed spontaneous afferent discharges and action potentials from intrafusal muscle fibres accompanied by contractions. Spontaneous discharges were also recorded from spindle in situ on the surface of an extensor muscle with intact nerve and vascular supply. The afferent discharge were probably recorded from intrafusal myelinated nerve branches. Spontaneous intrafusal contractions (0 [illegible]—0.4/sec) evoked single and repetitive afferent discharges. Most of the spontaneous afferent discharges (bursts [illegible] 3—6/sec) occurred independently of preceding action potentials from intrafusal muscle fibres and did not disappear when intrafusal contractions were abolished by tubocurarine or injury. The rate of afferent discharges increased with the initial elongation and with the temperature, whereas the rate of spontaneous intrafusal contractions remained unchanged. The transequatorial conduction rate of the spontaneous discharges from single intrafusal muscle fibres was 0.[illegible]4—0.38 m/sec (18—20° C) increasing with the fibre diameter. Conduction failed when the amplitude of the action potential was below critical size. In the pre-paralytical state high concentrations of d-T bocurarine prolonged the time course of the spontaneous intrafusal contractions and initiated bursts of afferent discharges.

Spontaneous afferent activity is not a common property of mechanoreceptors. When the sensory ending is surrounded by inexcitable structures it was either never observed (amphibian touch receptors, Lindblom 1962) or its occurrence was uncertain (pressure receptors in dogfish, O. Loewenstein 1956). In Pacinian corpuscles spontaneously firing receptors were found only occasionally (W. Loewenstein 1958).

The sensory endings of the muscle spindle are attached to another excitable structure, the intrafusal muscle fibres. Contractions of these fibres facilitate a maintained afferent resting discharge by stimulating the sensory endings on their surface (Katz 1961, Jahn 1966). In this respect the muscle spindles resemble the stretch receptors of lobster and crayfish which show background activity (Kuffler 1954).

Spontaneous afferent discharges of the spindle at rest were observed since the first electrophysiological studies of tension receptors in frog muscles (Adrian and Zotterman 1926, Matthews 1931). Buller, Nicholls and Ström (1953) assumed the activity to be due to thermal agitation noise in the nerve endings. They did not however consider the possibility that some of the random discharges might be evoked or facilitated by spontaneous changes in the mechanical state of the intrafusal muscle fibres. Increase of the rate of spontaneous afferent discharges with temperature was reported by Corda (1961).

During morphological studies of isolated frog muscle spindles (Jahn 1959) spontaneous intrafusal contractions were observed which were also noticed by Ottoson (1961). Recording of action potentials from the equatorial region allowed both the spontaneous afferent activity and the spontaneous discharges from intrafusal muscle fibres to be studied.

The aim of the investigations presented in this report was to analyse these two types of spontaneous activity with respect to the origin of the action potentials, the effects of the initial elongation of the preparation and of temperature on the discharge rates, their interactions, the conduction of the spontaneous action potentials from the intrafusal muscle fibres and the effect of d-Tubocurarine.

Material and method

P[illegible]paration. The muscle spindles were isolated from m. extensor longus digiti IV of *Rana temporaria*, more rarely of *Rana [illegible]culenta*. The extensor muscles in situ were 11—14 mm long, 15 per cent above equilibrium length. The muscle spindle was dissected over [illegible] length of 1—2 mm (Jahn 1959). The muscle spindles contained probably 2—3 intrafusal muscle fibres; the number was not controlled in histological sections.

After dissection of the spindle [illegible]ylon threads were attached to the tendons of the extensor muscle. The central part of [illegible]he dissection chamber (Fig. 1) was transferred to the [illegible]ecording [illegible]hamber and the preparation was mounted by connecting the nylon threads to two moveable glass rods (r_1 and r_2). The initial elongation was measured by [illegible] millimeter scale [illegible]m). The sciatic nerv[illegible] was kept floating in [illegible] corner of the experimental chamber or fixed with [illegible]ooden rod attached to the connec[illegible] tissue around the nerve.

T[illegible] study the muscle spindles [illegible] [illegible]ut [illegible] decapitated frog (13—20 g) was placed on [illegible] par[illegible]ffin bench. One leg was fixed with needles at the ankle and the toes for dissection of [illegible]he ex[illegible]ensor muscle. The bone and m[illegible]scles parallel with the m. extensor longus digiti IV wer[illegible] [illegible]emoved and care was taken to preserv[illegible] the nerv[illegible] and the [illegible]ascula[illegible] supply of the extensor muscle.

The effec[illegible] of denerv[illegible]tion on the spontaneous intrafusal contractions was [illegible]died. Frogs were anesthetized with [illegible] [illegible]rethane solution injected into the lymphatic sac ([illegible] 0.1 ml [illegible] fro[illegible] [illegible] [illegible]mm long portion of the sciatic nerv[illegible] was removed and the frogs were kept in [illegible] [illegible] containing 6 mg Na P[illegible]nicilline per liter. 2—7 day[illegible] after denervation the [illegible] [illegible]erals decapitated [illegible]nd the[illegible] muscle spindles were studied both in si[illegible] and after isola[illegible]ion.

Ringer solution. T[illegible] [illegible] [illegible]vestigat[illegible] whether the spontaneous intrafusal contra[illegible]tion[illegible] [illegible] [illegible]d muscle spindles were [illegible]nduced by [illegible]he ionic environment four types of Ringer [illegible] [illegible] were used (Table 1).

The pH wa[illegible] adjusted to [illegible]7.[illegible]4 by adding 1.6 ml 2.5 % N HCO$_3$ sol[illegible] [illegible] [illegible] mM before bubbling [illegible]h[illegible] [illegible]a[illegible] mixture of 99 % O$_2$ and 1 % CO$_2$. The colloid osmo[illegible] [illegible]pressure of Ringer solution [illegible] [illegible] [illegible]1[illegible] [illegible] 11 mm H$_2$O (Sten-Knudsen 1953 pp. 22—24 [illegible]b [illegible] [illegible] NaCl and [illegible]Cl$_2$ [illegible]e ionic composition of type I—III corresponded to the Ri[illegible] [illegible] [illegible] [illegible]solution used by Ottoson 1964 and 1965a). The increase in osmotic pressure [illegible] addition of glucose and dextran (type I) was negligible [illegible] [illegible] [illegible]ew of the effect of h[illegible] used [illegible] [illegible] solutions on afferent spindle act[illegible]vity (Ottoson 1965b).

Some of the results were reported in tw[illegible] short communications (Bo[illegible] [illegible] and Jahn (1937) [illegible]nd J[illegible]hn 1966.

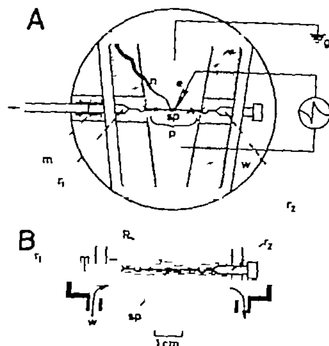


Fig. 1 Experimental chamber: A. from above. B side view p extensor muscle with both tendons and bundles of cut extrafusal fibres left. sp isolated muscle spindle. sciatic nerve with branches to the extensor muscle and the single afferent fibre to the spindle. r1 r2. movable glass rods. w water-cooled stage. m scale fo measurement of elongation macroelectrode for intrafusal recording g ground connection. R Ringer solution

Table I Ringer solution

Type	I	II	III	IV
NaCl	115 mM	115 mM	115 mM	112 mM
KCl	2.7 mM	2.7 mM	2.7 mM	2.5 mM
CaCl	1.8 mM	1.8 mM	1.8 mM	1.8 mM
glucose	0.2 g per liter	—	—	—
dextran	30 g per liter	—	—	—
agar*	—	—	2 per cent	—

Jahn (1966) used by Smith (1964a)
Special Agar Noble Difco Lab. Inc Detroit

Samples of the differen types of Ringer' solution were analyzed for heir content of K and Ca. There were no changes induced by the addition of dextran or Agar

The temperature of the Ringer bath was kept at 17–20 C and in four experiments at 24—25 C.

Recording Intrafusal recording was either extracellular or more rarely intracellular The tip diameter of the glass capillary microelectrodes was 0.3—0.5 μ. For extracellular recording electrodes were selected with an impedance of 4—8 MΩ they were filled ith 3 M NaCl solution. For intracellular recording the electrodes wer filled with 3 M KCl solution, the impedance was about 10 MΩ The reference electrode was an Ag-AgCl lectrode placed in the Ringer' bath. Insulated platinum wire 30 μ in diameter with bared p. was used to record from the surface of the spindle capsule or the surfac of thin extensor muscle directed ... or

The preparations were covered by mineral oil. The reference lectrode which was also of platinum, was placed on one of the tendons.

T record simultaneously with two microelectrodes they were mounted on the two arms of Zeiss micro-manipulator. One of the electrodes was moved straight downwards, the other at an angle arying with the distance between the two recording sites.

Stimulation. Evoked intrafusal contractions were obtained by stimulating the motor nerv through microelectrode (filled with 3 M NaCl solution) with rectangular pulses 0.1—0.3 msec in duration, delivered at rate of less than 1/sec.

The amplifiers were c. coupled, they had cathode follower input with an input impedance of 100 MΩ and an effecti capacity to ground of about 5μμF. The lower limiting frequency was 1 c/s, the upper 5—10 kc/s (3 db down). The amplified action potentials were displayed on double beam oscilloscope (DuMont, type 279). The amplitude and the duration of the action potentials were measured on sweep recordings (sweep lengths 8—30 msec). T determine the propagation elocity of the action potential along intrafusal muscle fibres, the sweep was triggered by the action potential (Buchthal, Guld and Rosenfalck 1957) recorded nearest to the sou t which the spontaneous impulse started.

The rate of the spontaneous discharges was determined on continuously moving film (15—30 cm/sec. Fairchild camera, Model 321 and Grass camera, Model C 4 F).

Results

W th the microelectrode within the perifascicular space of the muscle spindle (Fig. 2A and B) two types of spontaneous discharge were recorded from the equatorial region. According to their origin in the nervous and muscular part of the receptor they were denoted as n and m potentials. They differed in duration and in firing rate. Intrafusal recording could be maintained for several hours without change in the mean rate of discharges, though the amplitude of the action potentials often changed suddenly probably because a contraction of intrafusal muscle fibres changed the distance between the recording electrode and the gener ator" n and m potentials were also recorded with platinum lectrodes (platinum wire, 30 μ m diameter) f om the surface of the isolated muscle spindl in mineral oil (Fig. 2B) and in one experiment from a muscle spindle *in situ* on the surfac of a thin extensor muscle.

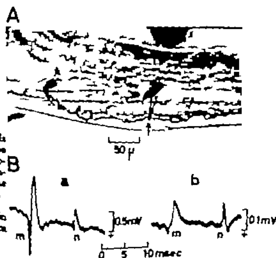


Fig. Spontaneous action potentials (m and potentials) from the equatorial region of an isolated neuromuscular spindle. A. Microelectrode inserted in the perifascicular space of the spindle marked by the arrow). B m nd "n discharges recorded ( ) by microelectrode with the spindle in Ringer solution, (b) by 50 platinum lectrode placed on the surfac of spindle immersed in mineral oil.

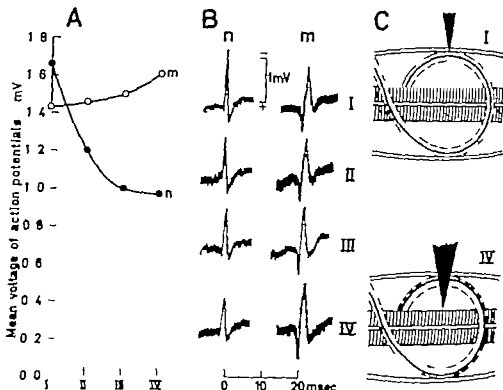


Fig 3. Change in action potential amplitude as the electrode was advanced from the capsule to the surface of the intrafusal muscle bundle (C) A Amplitude of and m potentials at positions of the microelectrode (I—IV) B and m potentials at the four positions. In "I" the electrode had just penetrated the spindle capsule in "IV" it was 15—20 μ inside in the central part of the perifascicular space.

Spontaneous afferent firing The n potentials were diphasic with a steep negative onset and a total duration of 2—3 msec. They were recorded in the equatorial and not in the polar region of the muscle spindle When the electrode was advanced in steps of about 5 μ from the capsule towards the centre of the perifascicular space, the amplitude was greatest (1—2 mV) after the microelectrode just had penetrated the spindle capsule and lowest (0.3—0.8 mV) close to the intrafusal muscle fibres (Fig 3) This is consistent with the assumpt on that the n potentials were recorded from intrafusal afferent nerve branches rather than from the sensory endings on the surface of the muscle fibres

Among "n potentials recorded from 32 spindles the polarity of the initial spike was negative in 28 and posit ve in 4 The firing of the n potentials was irregular the mean rate being 5—6/sec (4 spindles) at 17—19° C and equilibrium length. At 15 per cent stretch (length *in situ*) the rate was 6—9/sec at 17—19° C and increased to 10—11/sec at 24—25 C (Table II) The fluctuation of the afferent discharge rate amounted to 3 imp/sec both in the stretched and in the unstretched spindle and at 17 and 25 C the same as found by Buller Nicholls and Strom (1953) for rates between 7 and 163/sec (3.5 ± 0.25 imp/sec)

TABLE II. Rate[1] of spontaneous activity in isolated muscle spindles

Spindle no.	Afferent discharges ( )				Discharges from intrafusal muscle fibres (m)			
	mean interval (t_{mean} in msec)	S.D. (msec)	Rate (discharges per sec)	S.D.$_{rate}$	Mean interval (t_{mean} in msec)	S.D (msec)	Rate (discharges per sec)	S.D.$_{rate}$
				17—19 C				
1	104	28	9.6	2.8	2,290	1,350	0.4	0 4
2	130	32	8.0	2.0	2,340	1 410	0.4	0 4
3	144	48	7.0	2.6	2,430	1 440	0.4	0.4
4	165	62	6.1	2.7	3,290	2,310	0.3	0.5
5	132	42	7.6	2.7	2,470	933	0 4	0.2
				24—25 C				
6	93	31	10.7	4 1	3,430	1,000	0.3	0.1
7	97	32	10.3	3.8	2,900	1,290	0.4	0.2
8	94	27	10.6	3.3	4,060	1,940	0.3	0.1
9	95	28	10.5	3.4	2,680	1,090	0.4	0.2

Calculated from 100 "n and 20—25 "m potentials in each spindle.
Stretched 15 per cent above equilibrium length.

$$\text{S.D}_{rate} \; 1/2 \left(\frac{1}{t_{mean}-\text{S.D}_{interval}} - \frac{1}{t_{mean}+\text{S.D.}_{interval}}\right)$$

calculated according to Buller Nicholls and Ström (1953)

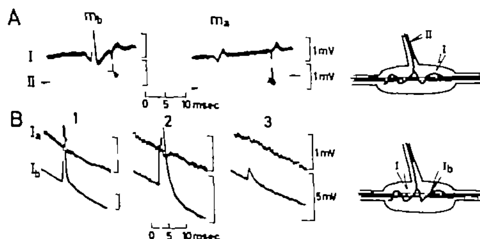


Fig 4 A Afferent discharges recorded from the equatorial region (I) and from the afferent nerv (II) evoked by spontaneous intrafusal contraction of single muscle fibre (m_b) or of two or three fibres in the same bundle (m_a) B Spontaneous afferent discharges from the equatorial region recorded extracellularly (I) and intracellularly (I b) at sites 150 apart. The large intracellular potentials (1 2) were also recorded extracellularly the fractional intracellular discharge (3) did not appear in the extracellular recording The position of the two microelectrodes is illustrated to the right.

"n discharges recorded in the equatorial region with two microelectrodes 150—250 μ apart occurred without measurable time delay. They were probably recorded from two different intrafusal nerve branches (cf Fig 4 in Jahn 1959) and originated from a common generator potential. Moreover the n" potentials were recorded without measurable time delay in the single afferent nerve fibre outside the spindle at a distance of 70—100 μ from the capsule (3 expts.) These nerve fibres had an axon diameter of about 11 μ (Jahn 1959) m potentials did not appear in the recording from the nerve even if when they were measured in the spindle, their amplitude was more than twice that of the afferent discharges recorded from the nerve, (Fig 4A)

Intracellular "n" potentials In 5 expts. the spontaneous afferent discharges from the equatorial region were recorded intracellularly probably from an intrafusal nerve branch. The amplitude of the potentials was 30—45 mV (Fig 4B) and declined gradually within 2—5 seconds probably due to changes in the electrode position during intrafusal contractions. Successive intracellular n potentials varied in amplitude by 25—30 per cent extracellular n potentials varied less than 10 per cent. In addition to the potentials of 30—45 mV there were intracellular discharges of 1—3 mV occurring at a rate of less than 1/sec. Simultaneous extracellular and intracellular recordings (2 expts.) at a distance of 150—170 showed that the extracellular n potentials occurred simultaneously with the intracellular ones except for the discharges of 1—3 mV which did not appear in the extracellular recording (Fig 4B)

ntaneous discharge f om the intrafusal muscl fibr s The m potentials were corded both from the equatorial region and from the polar regions of the muscle pindle (Fig 5A) indicating that they originated from the intrafusal muscle fibres. They were triphasic and had a amplitude of 0 8—2.0 mV (type a) and of 1.6—6 mV (type b) In both the amplitude was greatest when the electrode was close to the intrafusal muscle bundle and lowest when the capsule was just penetrated (Fig. 3) Each m discharge was associated w th an intrafusal contraction

Microscopial observations of pontaneous intrafusal contractions In the muscle spindle *in situ* with a diamete of less than 70 μ the rate of microscopically observed intrafusal contractions was 0 ? sec (3 expts., in 1 controlled by the recording of action potentials) Muscle spindles 60—65 μ in diameter in the equatorial region (probably containing two intrafusal muscl fibres, Jahn 1959) had a lower rate of spontaneous contractions than spindles of more than 70 μ with 3 intrafusal muscle fibres. Over an observation period of 1—2 min the maximum rate of spontaneous contractions or of action potentials was 0 4 sec, independent of hether the spindle was observed on the surface of an intact muscle or all the surrounding extrafusal muscle fibres had been removed.

In 6 isolated muscle spindles contracting at 0 3—0 4 sec the rate did not change if the spindle was stretched 40 per cent abov equilibrium length. At 50—70 per cent stretch the contractions could no longer be discriminated.

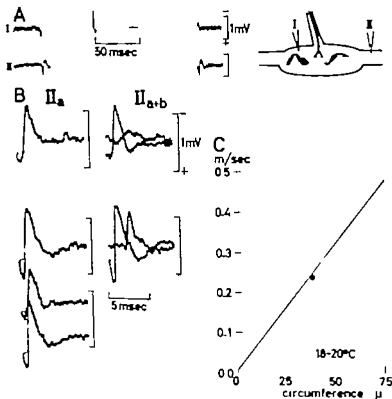


Fig 5 Conduction of spontaneous discharges over intrafusal muscle fibres. A Simultaneous recording from the equatorial region (I) and the polar region (II) B Sequence of action potentials recorded from one polar region. (IIa) half of the potentials had 30 per cent lower amplitude. Recording from both polar regions (IIa and b) 570 μ part showed propagation across the equatorial zone as long as the potentials had full size. The sweep of the oscilloscope was triggered by the initial part of the first action potential. C Conduction elocity of action potentials from intrafusal muscle fibres as function of the fibre circumference the straight line is extrapolated from findings on extrafusal muscle fibres (Håkansson 1956)

Rat of m potentials The a erage rate of m potentials was the same at 17—19° C as at 24—25 C and in all four types of Ringer sol tion but the discharge occurred more regularly at the higher temperature (S D 0 1—0.2 impulses sec at 17—19° C S.D 0.4 imp sec Table II)

The rate of m potentials recorded from isolated muscle spindles remained un changed for 6—7 hrs at 17—20° C at rates of 0 2—0 4 sec Isolated muscle pindles kept in Ringer s Agar for 24—26 hrs (over night at 2 C) discharged spontaneously t rate of 0 1—0 sec when brought back to room temperature (3 spindles)

C nl actions ac ompanied by m" pot ntials type Potentials of type a had pike sizes between 0.8 and 2.0 mV and duration f 3—4 msec They were recorded from all spindles w th spontaneous intrafusal co tractions and associated w th the contraction of one intrafusal muscle fibre In one third of the muscle spindles m potentials of type occurred alternatively w th a 30—40 per cent reduced spike size and duration of 4—5 msec.

Contractions accompanied by "m" potentials type b In 7 muscle spindles with a diameter exceeding 70 μ the intrafusal muscle bundle occasionally contracted as a whole. These contractions were accompanied by "m" potentials with 2—3 times greater amplitude and a duration of 4—5 msec (denoted type b) than the "m" potentials type a. During observation periods of 5—10 seconds they often comprised one third to half of the "m" discharges, observed over longer periods they could fail to occur for 2 min.

The movement artefact indicated that the mechanical responses associated with the large "m" potentials had a similar duration as responses associated with "m" potentials type a (Figs. 6 and 7 and Jahn — in preparation —)

Denervation In three frogs studied 2, 3 and 7 days after section of the sciatic nerve intrafusal contractions occurred *in situ* and in isolated spindles at a rate of less than 5 min (microscopical observations)

d Tubocurarine abolished the spontaneous intrafusal contractions.

This was observed both when muscle spindles were studied microscopically *in situ* after injection of d-Tubocurarine into the lymph sac (100—700 μg/frog) and in isolated muscle spindles. The Ringer' bath was replaced by Curare-Ringer' (5—15 μg d-Tubocurarine per ml) or 5—10 times higher concentration of d-Tubocurarine was added by drop-infusion (Table III). The concentrations of d-Tubocurarine applied do not represent minimum concentrations.

The contractions reappeared when the Curare-Ringer was exchanged with ordinary Ringer's solution. Both decline and recovery of the spontaneous contractions occurred slowly (15—80 min, Table III) as observed microscopically (Buchthal and Jahn 1957) and by recording of the action potentials during application and [illegible] of the drug (Jahn 1966). d-Tubocurarine acted in lower concentrations [illegible] the surrounding extrafusal muscle fibres had been removed from the spindle, probably because of faster diffusion of the drug

Effect of injury The rate of intrafusal discharges declined suddenly or the spontaneous contractions observed microscopically disappeared when the spindle was injured by dissection, by movement of an electrode tip during insertion or by a strong intrafusal contraction

Rate of conduction Simultaneous recordings from both polar regions demonstrated that "m" potentials were propagated across the equatorial region (Fig 5). The discharges started in one spindle pole. The rate of conduction varied in 5 spindles (18—20 °C) between 0.24 and 0.38 m sec (Table IV, Buchthal and Jahn, 1957) increasing with the diameter of the fibres, which varied only little in a given spindle. The conduction rates of spontaneous discharges of the intrafusal muscle fibres were the same as for evoked potentials in *Xenopus laevis* (Smith 1964) and agreed with those predicted from the rectilinear relationship between circumference and velocity obtained for isolated extrafusal twitch fibres (Håkansson 1956, Buchthal and Sten-Knudsen 1959)

The rates previously given (Buchthal and Jahn 1957) were somewhat higher probably because the observation period was shorter

TABLE III. The effects of d-Tubocurarine on intrafusal contractions of frog muscle spindles.

Preparation	Method of application	Concentration of d-Tubocurarine (μg/ml)	Abolished min after Tubocurarine	Recovered min after Ringer
		Spontaneous contractions		
Isolated muscle spindle[1] (5 spindles)	Exchange of 50 ml solution	5—15	15—20	about 60
Muscle spindle surrounded by 4—6 extrafusal fibres[2] (4 spindles)	Drop infusion (2.5—3 ml/min for 5 min)	50—100	30—40	60—80
		Evoked contractions		
Muscle spindles in situ m. gastrocnemius[3]	Exchange of bath (80 ml)	70—150	20—30	

[1] Buchthal and Jahn (1937)
[2] Jahn (1966)
[3] Hensch and Schulte (1958a)

TABLE IV. Conduction of impulses along intrafusal muscle fibres of frog and toad muscle

Fibre diameter μ[1]	Conduction rate m/sec	Conduction distance (transequatorial) μ	Temperature	Species
	Spontaneous discharges			
13	0.24	490	18—20 °C	Rana temporaria
13	0.29	740		
14	0.28	630		
14	0.32	910		
16	0.38	570		
	Evoked discharges			
13—16	0.43, 0.33, 0.31	500	19—23 °C	Xenopus laevis
	0.64 (1 spindle)	9,000	17 °C	Rana temporaria

[1] measured both in the equatorial and polar region and taken as mean (Jahn 1959)
[2] Buchthal and Jahn (1937)
[3] Smith (1964a and b)
[4] Katz (1949)

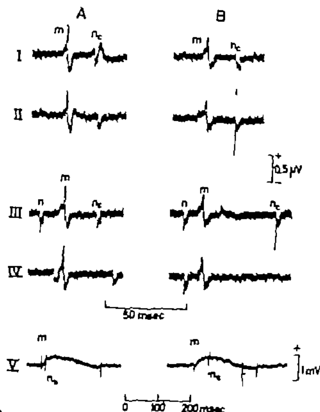


Fig. 6. Interaction between the spontaneous n and m potentials I and II Spontaneous intrafusal contractions, accompanied by muscle fibre potentials (m) of ordinary size (A) and of 30 per cent smaller size (B) Both evoked afferent responses (n_c) after latency of 18—20 msec. III and IV A spontaneous afferent discharge ( ) preceding m potential of ordinary size by 14 msec did not impair subsequent afferent response (n_c) Spontaneous afferent discharges ( ) preceding m potentials by 5—10 msec delayed (A IV and B III) or prevented (B IV) the subsequent afferent response (n_c) B V Delayed afferent discharge (n_c) as occasionally evoked by intrafusal contractions associated with a m potential of reduced size. The contractile changes (movement artefact, A V and B V) lasted about 200 msec. Note the reversed polarity in these recordings.

Non-conduct d re ponse In two of the spindles in which transequatorial conduction was studied (Table IV) m potentials type a occurred th reduced amplitude These action potentials were not propagated. Whether action potentials with reduced and with normal spike size arose in the same or in diff rent fibres could not be decided

Interaction betw spontaneous m and n discha ges Each discharge from a single intrafusal muscle fibre was followed by an afferent discharge w th a delay of 11—25 msec, varying from spindle to spindle However in a gi en spindle and at a given recording site the delay varied less than ± 2 msec (Fig 6 I and II) m potentials with 30—40 per cent reduced amplitude probably associated with local contractions, also elicited an afferent response occasionally with latency of more than 25 msec (Fig 6)

Contractions of single intrafusal muscle fibres evoked by stimulation of the motor nerve (3 expts.) were followed by an afferent discharge after a similar delay as that after a spontaneous contraction. The first afferent discharge after the regularly occurring response during the contraction (duration about 200 msec. Fig 6 V) occurred in about half the spindles at an interval of 12—80 msec i.e. at a shorter interval than the a erage for the spontaneous afferent acti ity (Table II) indicating

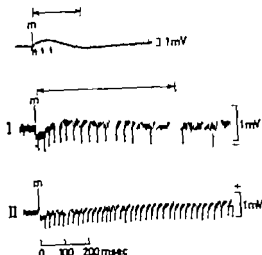


Fig. 7 Repetitive afferent firing. A Three afferent responses (marked by the arrows) evoked by spontaneous contraction of 2 or 3 intrafusal muscle fibres. The contractile changes (movement artefact) lasted about 200 msec. "m" action potential from intrafusal muscle fibres. B Bursts of afferent responses evoked by spontaneous contractions of single intrafusal muscle fibres occurring during the decline of the contraction rate after exposure to high concentrations of Curare (300 µg Tubocurarine per ml Ringer). I Burst of 16 discharges associated with mechanical deformation of about 600 msec duration (as judged from the movement artefact). II Onset of burst of afferent discharges which lasted about 1 1/2 sec.

either that a second discharge was occasionally evoked or that spontaneous firing was facilitated.

When a spontaneous afferent discharge ("n") preceded a "m" potential, type a, by 5—8 msec the afferent response to the spontaneous intrafusal contraction (n) was delayed. It failed to appear when the interval between the spontaneous "n" and the "m" potentials was less than 5 msec. "m" potentials with reduced amplitude were not accompanied by an afferent response to the intrafusal contraction unless the interval between the preceding "n" discharge and the "m" potential exceeded 10—11 msec, indicating that these contractions elicited a weaker stimulus to the sensory endings (Fig. 6 III and IV).

Repetitive firing. Most "m" potentials type b were followed by 1—3 afferent responses. In 3 spindles every contraction was followed by 2—3 discharges, the first 6—10 msec, the second 20—30 msec and the third 30—40 msec after the "m" potential (Fig. 7 A). In two other spindles the "m" potential was followed by 1—2 and occasionally by bursts of 6—7 afferent discharges with intervals of 10—20 msec between the spikes. The increased number of discharges after large "m" potentials (type b) and the larger movement artefact during the contraction indicate that these potentials were associated with contractions of at least 2 intrafusal muscle fibres, the greater deformation of the sensory ending acting as a stronger stimulus to the sensory ending (cf. Fig. 6 V and Fig. 7 A).

Repetitive firing after spontaneous "m" potentials type was observed only after drop infusions of high concentrations of d-Tubocurarine (100—300 µg/ml) during the decline of the discharge rate until the "m" potentials disappeared completely or during the recovery of the spontaneous contractions in normal Ringer until the "m" potentials resumed their original rate. The maximum number of repetitive discharges increased with the d-Tubocurarine concentration: there were discharges in 100 µg/ml, 15—16 in 300 µg/ml and once 0 discharges 300 µg/ml

(Fig 7 B) As judged from the movement artefact the contractions associated with repetitive afferent firing lasted at most 600 msec (Fig 7 B) Repetitive afferent firing due to preparalytic excitation of intrafusal motor end plates has been reported after application of gallamine triethiodide (Flaxedil® Henatsch and Schulte 1958 b)

Spontaneous contractions and rate of afferent discharges When the rate of intrafusal contractions was low or contractions were abolished by injury to the intrafusal muscle fibres the spontaneous afferent firing persisted at a lower rate (reduction to 50—70 per cent when the contractions were completely abolished) The rate of spontaneous afferent discharges was reduced to half when the spontaneous intrafusal contractions were abolished by d Tubocurarine (Jahn 1966) Assuming that the drug does not affect the sensory endings, each spontaneous contraction is responsible for 9—10 afferent discharges though only 1 or occasionally 3 of them occurred in a fixed temporal relation to the contraction.

Discussion

A Spontaneous intrafusal contractions

Extrafusal muscle fibres *in situ* or isolated did not contract spontaneously In some fibres of the ileofibular muscle of the frog intracellularly recorded spontaneous discharges were occasionally seen after cessation of rhythmical stimulation (Shamarina 1962) In muscle spindles of *Xenopus* spontaneous activity occurred only after cessation of repetitive stimuli to the motor nerve (Smith 1964 b)

The spontaneous contractions in intrafusal muscle fibres of the frog occurred in all carefully dissected preparations without preceding stimulation. The following findings make it likely that these contractions are a physiological phenomenon (i) they occurred both in isolated muscle spindles, muscle spindles in intact muscles and in muscle spindles *in situ* (ii) they persisted 24—26 hours in isolated preparations (iii) they occurred in a Ringer's solution of the same composition as used in muscle spindles of *Xenopus* (Table I, type IV) (iv) the spontaneous activity was reversibly abolished by d Tubocurarine in concentrations similar to those which abolish evoked intrafusal contractions (Henatsch and Schulte 1958 a, Table III)

The effect of d Tubocurarine supports the assumption that the spontaneous contractions originate from an end plate activity analogous to the miniature potentials in the motor end-plates of extrafusal muscle fibres (Fatt and Katz 1952) This activity appeared to be due to the liberation of small amounts of acetylcholine possibly stored in vesicles of 400—600 Å in the terminal axoplasm (Birks, Huxley and Katz 1960) The same vesicles were also found in the intrafusal myoneural junction of frog (Karlsson and Andersson-Cedergren 1966) Small amounts of transmitter are more apt to produce visible contractions in the thin intrafusal muscle fibres than in the larger extrafusal fibres. Moreover the large contact areas of the motor ending (150—200 µ) found in intrafusal muscle fibres (Gray 1957 Karlsson and Andersson-Cedergren 1966) are compatible with this interpretation as is the absence of an interposed basement membrane noticed in intrafusal motor end-plates both, of

the frog (Karlsson and Andersson-Cedergren 1966) and of the rat (Merrillees 1960) resulting in a tight contact between nerve and muscle plasma.

In agreement with the smaller diameter the conduction velocity over intrafusal fibres was about 10 times slower than over extrafusal ones. That the diameter of the intrafusal muscle fibres is still smaller (4—5 μ) in the 100 μ long reticular zone (Karlsson, Andersson-Cedergren and Ottoson 1966) is compatible with an unchanged over-all conduction rate. The velocity of about 0.3 m sec and the duration of the negative phase of the action potential of 1.5—2 msec imply that the depolarization extends over a length of 450—600 μ. Hence the 100 μ wide reticular zone does not need to reduce the conduction velocity.

The conduction rate over the intrafusal fibres is hardly affected by the 400 Ω cm² resistance of the capsule measured by Smith (1964 a) in *Xenopus laevis*. The longitudinal resistance of the perifascicular space is small as compared with that of an intrafusal muscle fibre and no significant slowing of conduction can be expected (Katz 1948). Smith (1964 a) stimulated the intrafusal muscle fibres indirectly and evoked action potentials of complex shape, the initial deflection being negative. He attributed this complexity to a reduced safety factor in the reticular region of the fibre. Since complexity was not observed in the spontaneous action potentials of intrafusal frog muscle fibres, it seems more likely that the action potentials recorded by Smith (1964 a) are the summated response of two intrafusal muscle fibres, one end plate lying close to the recording electrode, the other at a distance of about 1 mm. The change in action potential shape seen with paired or repetitive stimuli (Smith 1964 a) would then reflect a difference in refractory period of the two fibres.

The afferent response evoked by spontaneous intrafusal twitches or following stimulation of the motor axon is probably due to stimulation of the sensory terminations within the reticular zone, which is almost devoid of contractile elements and therefore likely to be stretched during contraction of the fibre (Katz 1961). The fixed temporal relation between an intrafusal contraction and the afferent response has previously been reported in muscle spindles of toads (Eyzaguirre 1957) and in muscle spindles of cats (Boyd 1966).

In toads two types of intrafusal muscle fibres were distinguished which differed in fiber diameter and in duration of the evoked action potential (Smith 1964 a and b). The duration of the spontaneous action potentials (type) was the same whether they had normal or reduced amplitude and the difference in amplitude and ability to conduct cannot be considered evidence of two types of fibres. Twitch-like local contractions evoked by stimulation of the motor nerve were also observed in intrafusal fibres of mammalian muscle spindles (Boyd 1966).

B. Spontaneous afferent discharges

The spontaneous nerve discharges recorded intrafusally in the equatorial region could originate either in the sensory end-bulbs or in intrafusal nerve branches. They were probably recorded from myelinated nerve branches because their amplitu

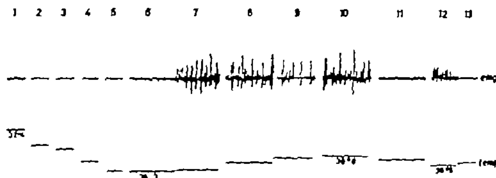
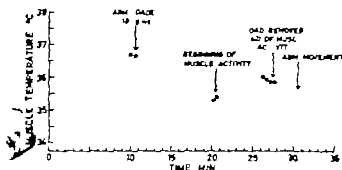


Fig. 1 Mom. 1 and 2 the beginning and the end of the first 10 min period when the arm is hanging freely Mom 3—6 the following 10 min arm still hanging ertically weight of 12 kg is pplied to the hand. I mom. 7 an inconsiderable flexion at the elbow-joint was performed in order to induce the muscle to acti ty The arm and the weight was kept in this position in 8 min, mom 7—10 Mom 11 1 mm after the weight was taken away Mom 12 the last one of ten oluntary contractions of the elbow-flexor muscles Mom. 13 immediately after these ontractions



F g 2 The progressiv changes in muscle temperature during an experiment (Subj B B)

work for quite a long time, up to 15 min or more, with the same blood supply as when the arm is hanging freely inacti ely and without load. However as soon as the muscle becomes acti e, the circumstances changes and the need of oxygen demands a higher blood supply Consequently the temperature also rises. Th reason for the temperature to increase further when the load on the arm was removed after several minutes of isometric muscle contraction, must be the fact that the intra muscular pressure responsible for the partial occlusion of the blood supply during the contraction decreased as soon as the load was dropped

In such working positions and working movements where ligaments nd capsules can take over an important part of the static muscle work, the need of oxygen is less than if the work is produced only by the muscles as the metabolism in the connective tissue is considerably lower From these observations it is evident that work can be produced which is not reflected in the pulse rate oxygen consumption or increased blood supply

References

BARCROFT H, B GREENWOOD and R F WHELAN, Blood flow and venous O_2 saturation during sustained contractions of the forearm muscles. *J Physiol.* (Lond.) 1963 *168*, 848—856.
BARCROFT H and J L E MILLEN, The blood flow through muscle during sustained contractions. *J Physiol.* (Lond.) 1939 *97* 17—31.
BASMAJIAN J V *Muscles Alive* Williams and Wilkins, Baltimore. 1962.
BASMAJIAN, J V Weight bearing by ligaments and muscles. *Canad. J Surg* 1961 *4* 166—170.
HUMPHREYS, P W and A R LIND, The blood flow through active and inactive muscles of the forearm during sustained hand grip contractions. *J Physiol.* (Lond.) 1963. *166*. 120—135

Acta physiol. scand. 1968. 72. 370—377

From the Department of Physiology Faculty of Medicine Karolinska Institutet, Stockholm Sweden

Effect of Reserpine on the Noradrenaline Content of the Vas Deferens and the Seminal Vesicle Compared with the Submaxillary Gland and the Heart of the Rat

By

NILS O. SJÖSTRAND and GÖRAN SWEDIN

Received 11 July 1967

Abstract

SJÖSTRAND, N. O. and G. SWEDIN. *Effect of reserpine on the noradrenaline content of the vas deferens and the seminal vesicle compared with th submaxillary gland and the heart of the at.* Acta physiol. scand. 1968. 72. 370—377

A low dose (50 µg/kg) of reserpine was used in order to elucidate the possible difference in rate of noradrenaline depletion between the male genital organs, the submaxillary gland and the heart of the rat. Reserpine reduced the noradrenaline content of the male genital organs slowly and less completely than that of the submaxillary gland and the heart. There were differences in reserpine sensitivity between innervated and decentralized genital organs. The decentralized submaxillary gland, however showed tendency to less rapid initial depletion en compared with the innervated gland. It is suggested that the differences in depletion rate could be due to different properties of the short adrenergic neurons innervating the genital organs when compared with the ordinary long ones to the heart and the salivary gland. Another contributing factor may be differences in blood flow through the organs. It is further suggested that the genital organs has lower sympathetic nerv impulse frequency in live

According to recent findings the vas deferens and the accessory male genital glands of the rat and other mammals are innervated by short adrenergic neurons, i.e. neurons having their cell bodies located close to the target organs (*cf.* Sjöstrand 1965). Recent evidence has also been presented indicating that the catecholamine containing granules of short adrenergic neurons in several respects differ from those of ordinary long ones (Euler and Lishajko 1966, Stjärne and Lishajko 1966). This indicates that the short adrenergic neurons form a specific entity of adrenergic tissue (Owman and Sjöstrand 1966).

It has been established by numerous investigators that reserpine causes a depletion of catecholamines from adrenergic tissues (*cf.* Carlsson 1965). It therefore seemed to be of interest to compare the effect of reserpine on the noradrenaline content of organs innervated by short adrenergic neurons, such as the as deferens and the

seminal vesicle, with organs innervated by ordinary long adrenergic neurons, like the heart and the submaxillary gland, which are frequently used in studies concerning adrenergic mechanisms.

Many authors (*cf* Sedvall 1964) have suggested that the reserpine-induced noradrenaline depletion is more rapid and complete in innervated organs than in organs deprived of their connection with the central nervous system. It therefore also seemed to be of interest to compare the effect of reserpine on the noradrenaline content of innervated as well as decentralized organs.

Materials and methods

32 male Sprague-Dawley rats, initially weighing about 300 g were used in this study. All animals were submitted to unilateral cervical sympathetic decentralization and section of the hypogastric nerves. The submaxillary gland and the genital organs of the untreated side served as controls. In order to avoid systematic anatomical aberrations each experimental group of two animals consisted of one animal decentralized on the left side and the other on the right side. In order not to cause any gross changes in the conditions of the animals the nerve sections were performed 3—4 weeks before the experiment.

Surgical treatment. After pretreatment with atropine (1 mg/kg i.m.) the rats were anesthetized with nembutal, supplemented when necessary with ether. The cervical sympathetic trunk and the vagus nerve in the neck were sectioned and about 1 cm of the nerves was removed. With the animal still under anesthesia the abdomen was opened and the hypogastric nerve cut on the same side as the cervical sympathetic trunk. After removal of about 2 cm of the hypogastric nerve the abdomen was closed. The result of the surgical procedures was controlled by the ptosis and enophthalmus which developed a few hours postoperatively on the treated side. At the time of killing the vas deferens and the seminal vesicles on the denervated side were distended and filled with spermatozoa and secretion products.

Reserpine. The animals were divided in 5 groups. 8 animals in one group served as untreated controls. The other groups of 6 animals each were injected with reserpine (Serpasil®) 50 µg/kg s.c. The drug was used in a concentration of 1 mg/ml and diluted in the commercial solvent.

Extraction procedure. After intervals of 2, 6, 12 and 24 hrs respectively the animals were sacrificed under ether anesthesia. The submaxillary glands, hearts, vasa deferentia and seminal vesicles were quickly removed and weighed. With exception of the hearts, contralateral organs of two animals were pooled and homogenized with an Ultra Turrax apparatus. Extraction was carried out in a total volume of 20 ml of ice-cold 0.4 N perchloric acid. The extracts were centrifuged at 9 000 rpm for 15 min at 0° C, whereafter the supernatant was adjusted to pH 3.8 with 2 N KOH. The extracts were kept frozen for not more than 2 weeks before estimation of the noradrenaline. The extracts were then gently thawed and adjusted to pH 8.2 with 1 N NaOH before adsorption on alumina columns. Elution was performed with two 5 ml portions of 0.25 N acetic acid. The noradrenaline was determined according to Euler and Lishajko (1961) and is expressed as µg free base per g of wet tissue weight.

Results

The results are shown in Table I. As seen from the table there are no marked differences in the weights of the animals from different groups, nor in the weights of the organs with one exception, the submaxillary glands. The weights of the sympathetically decentralized submaxillary glands were in all cases reduced by about 15 per cent. This is in accordance with previous findings (*cf* Ohlin 1966) and is probably due to a reduction in glandular parenchyma. This is probably also the reason why there is a higher noradrenaline concentration in the decentralized submaxillary

A slight difference was obtained from the 24 hrs group when compared with the untreated controls (0.05>p>0.01).

glands than in the innervated ones. The total amounts of noradrenaline in the glands are therefore also presented in the table. A slight tendency to weight loss during the first 2 hrs of reserpine treatment is also seen in the submaxillary glands without any difference between innervated and decentralized organs.

Fig 1 shows per cent remaining noradrenaline after different time intervals after reserpine. From the figure it is obvious that reserpine reduces the noradrenaline content of the vas deferens and the seminal vesicle more slowly and to a lesser extent than that of the heart and the submaxillary gland. This is a systematical difference seen at all time intervals. The remaining amounts of noradrenaline are about 40, 25, 15 and 10 per cent respectively

The effect of decentralization seems to be slight if any on the genital organs. For the submaxillary gland there seems to be a tendency to a slower initial depletion in the decentralized organs. After 2 hrs there is about 42 per cent left in the decentralized organs but only 28 per cent in the innervated ones. The minimum values seem, however to be about the same for innervated and decentralized organs.

The difference between the organs is further illustrated when presented in a semilogarithmic system (Fig. 2) The noradrenaline values are here expressed as total amounts per pair of organs. It is obvious that there are two different phases of noradrenaline depletion in the submaxillary gland and also, but less marked, in the seminal vesicle, while the vas deferens overtly lacks the rapid initial phase. The rapid phase of the submaxillary gland depletion has a half time of about 1 hr with a difference of about 30 per cent between innervated and decentralized organs. The l vesicle takes an intermediate position with about 3 hrs while the as deferens the same order of half-time, 9—12 hrs, before and after the 2 hrs point. During the slow phase the curves run roughly parallell with half times in the same range of hours for all the organs.

Discussion

The present results show that reserpine, in the dose used reduces th noradrenaline content of the male genital organs more slowly and to a lesser extent than that of the heart and the submaxillary gland of the rat. It has also been shown that there are no obvious differences in reserpine sensitivity between innervated and decentralized genital organs, while there is a tendency to slower depletion in the decentralized submaxillary gland when compared with the innervated organ. Further it is shown that the vas deferens lacks the rapid initial phase of depletion seen in the other organs when this low dose of reserpine is used.

It is well known that reserpine depletes the catecholamines from various tissues (Carlsson and Hillarp 1956, Holzbauer and Vogt 1956 and others, cf Carlsson 1965) It is also known that the rate of noradrenaline depletion aries with the dose of reserpine (Carlsson t al 1957 Bertler 1961 and others) In n earlier study (Sjöstrand 1962) it was shown that no measurable amounts of noradrenaline ere left in the vas deferens and the seminal esicl of the guinea pig after two da of treatment with reserpine (0.5 and 1 5 mg kg) Moreover a hea y dose of reserpine

Table I Effect of reserpine on the noradrenaline content of different organs, sympathetically

	Animal no.	Body weight (kg)	Vas deferens				Seminal vesicle	
			Weight of pair of organs (g)		Noradrenaline (μg/g)		Weight of pair of organs (g)	
			Innerv	Decentr	Innerv	Decentr	Innerv	Decentr
Controls	1	0.40	0.17	0.13	12.9	16.6	0.58	0.49
	2	0.40						
	3	0.39	0.12	0.12	18.5	17.9	0.43	0.50
	4	0.40						
	5	0.41	0.13	0.14	17.2	14.9	0.49	[illegible]
	6	0.37						
	7	0.40	0.11	0.13	17.5	16.5	0.52	0.50
	8	0.39						
Mean		0.40	0.13	0.13	16.5	16.5	0.51	0.50
Reserpine 2 h	9	0.38	0.14	0.11	11.9	11.7	0.50	0.50
	10	0.38						
	11	0.40	0.14	0.15	14.3	15.5	0.52	0.61
	12	0.38						
	13	0.39	0.13	0.14	15.6	15.4	0.49	0.51
	14	0.38						
Mean		0.39	0.14	0.15	13.9	15.5	0.50	0.54
Per cent of contr					84	82		
Reserpine 6 h	15	0.40	0.12	0.13	11.2	9.4	0.56	0.59
	16	0.38						
	17	0.34	0.14	0.13	9.6	9.1	0.50	0.49
	18	0.33						
	19	0.42	0.14	0.15	12.5	13.9	0.56	0.53
	20	0.37						
Mean		0.37	0.13	0.14	11.1	11.5	0.54	0.54
Per cent of contr					67	70		
Reserpine 12 hrs	21	0.38	0.13	0.11	4.5	5.6	0.54	0.48
	22	0.40						
	23	0.39	0.12	0.13	7.4	7.4	0.50	0.47
	24	0.36						
	25	0.37	0.16	0.15	5.5	6.8	0.56	0.53
	26	0.40						
Mean		0.38	0.14	0.13	5.8	6.6	0.53	0.50
Per cent of contr					35	40		
Reserpine 24 hrs	27	0.40	0.13	0.12	5.3	5.8	0.40	0.44
	28	0.40						
	29	0.34	0.14	0.12	8.5	9.2	0.56	0.48
	30	0.34						
	31	0.34	0.12	0.13	6.0	6.4	0.55	0.53
	32	0.38						
Mean		0.36	0.13	0.12	6.6	7.1	0.50	[illegible]
Per cent of contr					40	43		

innervated and decentralized

Noradrenaline (µg/g)		Submaxillary gland: Weight of pair of organs (g)		Submaxillary gland: Noradrenaline (µg/g)		Submaxillary gland: Total NA per organ (µg)		Heart: Weight (g)	Heart: Noradrenaline (µg/g)
Innerv	Decentr	Innerv	Decentr	Innerv	Decentr	Innerv	Decentr		
1.52	0.91	0.69	0.63	1.04	1.03	0.72	0.65	1.19 1.10	0.77 0.89
1.33	0.96	0.70	0.57	0.87	1.06	0.61	0.61	1.20 1.30	0.68 1.11
1.13	1.44	0.68	0.60	0.73	1.49	[illegible]	0.90	1.26 1.08	0.77 1.03
1.12	0.99	0.68	0.59	0.82	1.16	0.56	0.68	1.15 1.03	0.90 0.63
1.27	1.08	0.69	0.60	0.87	1.19	0.60	0.71	1.16	0.85
0.58	0.63	0.56	0.48	0.19	0.57	0.10	0.18	1.10 1.16	0.59 0.55
0.83	0.35	0.60	0.51	0.29	0.54	0.17	0.27	1.12 1.11	0.34 0.42
0.94	0.95	0.67	0.53	0.23	0.58	0.16	0.31	1.20 1.20	0.32 0.55
0.78	0.64	0.61	0.51	0.24	0.50	0.14	0.25	1.15	0.57
61	59			28	42				44
28	0.32	0.73	0.56	0.08	0.15	0.06	0.09	1.17 1.03	0.21 0.18
34	0.26	0.63	0.52	0.16	0.20	0.10	0.10	1.27 1.10	0.19 0.23
0.88	0.60	0.68	0.53	0.23	0.41	0.15	0.22	1.34 1.51	0.41 0.25
0.50	0.39	0.68	0.54	0.16	0.25	0.10	0.14	1.17	0.25
39	56			18	21				29
0.21	0.16	0.82	0.58	0.04	0.10	0.03	0.06	1.02 1.17	0.22 0.07
0.19	0.12	0.68	0.56	0.05	0.11	0.03	0.06	1.24 1.15	0.13 0.12
0.32	0.63	0.39	0.37	0.14	0.06	0.09	0.03	1.10 1.12	0.15 0.09
0.24	0.30	0.70	0.57	0.08	0.09	0.05	0.05	1.13	0.13
19	28			9	8				15
0.13	0.15	0.76	0.69	0.09	0.09	0.07	0.06	1.14 1.22	0.08 0.13
0.40	0.44	0.70	0.58	0.16	0.24	0.11	0.14	1.17 1.13	0.24 0.27
0.20	0.22	0.68	0.58	0.08	0.09	0.05	0.05	1.04 1.21	0.08 0.08
0.24	0.27	0.71	0.62	0.11	0.14	0.08	0.09	1.15	0.15
19	25			13	12				18

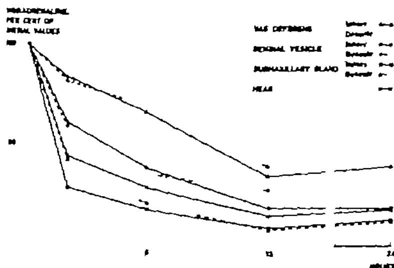


Fig. 1. Noradrenaline content of the innervated and decentralized vas deferens, seminal vesicle, heart and submaxillary gland of the rat at different time intervals after reserpine (50 μg/kg s.c.) expressed as per cent of initial values (μg/g wet tissue weight). Symbols see fig.

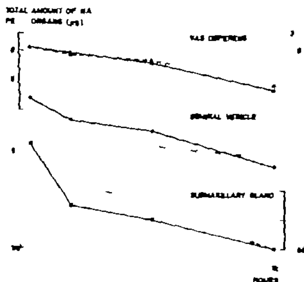


Fig. 2. Noradrenaline content of the innervated and decentralized vas deferens, seminal vesicle and submaxillary gland of the rat at different time intervals after reserpine (50 μg/kg s.c.). The noradrenaline content is expressed as total amount (in μg) per pair of organs and is plotted on a logarithmic scale. Symbols, see Fig. 1

(5—10 mg/kg) causes a complete disappearance of the specific catecholamine fluorescence in the rat vas deferens within 3 hrs (Norberg 1965). In this study, therefore, a low dose of reserpine was chosen in order to elucidate the possible difference in reserpine sensitivity between organs with different kinds of adrenergic innervation. It is already known that the heart is very sensitive to reserpine while the adrenal medulla is less sensitive. The brain takes an intermediate position. Other peripheral adrenergic tissues examined show about the same sensitivity to reserpine as the heart (Bertler 1961).

The depletion of noradrenaline from skeletal muscle after reserpine has been shown by Sedvall (1964) (see also Carlsson 1965) to show two distinct phases, the initial one fast and the second more slow The present results indicate the same kind of noradrenaline depletion from the submaxillary glands but a different one from the vas deferens. The explanation of the lower initial depleting rate after reserpine in the genital organs is at present obscure. It is, however tempting to suggest that this is due to some property of the special short adrenergic neurons innervating these organs (Falck, Owman and Sjöstrand 1965 Owman and Sjöstrand 1965 Sjöstrand 1965) In this respect recent findings of Euler and Lishajko (1966) are of interest. They found that the release rate of noradrenaline from isolated granules of the seminal vesicle and the vas deferens of the bull was several times lower than that of granules from splenic nerves, indicating different properties of the transmitter granules.

The intermediate position of the seminal vesicle with respect to the initial depleting phase could be due to the fact that relatively less of the noradrenaline in this organ is derived from short adrenergic neurons. From the histological picture it seems to be about the same amount of blood vessel innervation in the vas deferens and the seminal vesicle, whereas the smooth muscle innervation is far more abundant in the vas deferens. Anyhow the present findings support the view that the short adrenergic neurons constitute a specific entity of adrenergic tissue (*cf* Owman and Sjöstrand 1966)

Another factor which may be of some importance in explaining the differences in depletion rates between the examined organs is the blood flow through the organs, may affect the initial tissue concentrations of reserpine. Th genital organs have probably lower perfusion rates than the heart and also the salivary gland (Kopin, Gordon and Horst 1965) However it should be kept in mind that, in the present report, the heart although having a very high perfusion rate had a slower depletion rate than the submaxillary gland. Thus differences in blood perfusion between the organs are probably not the sole explanation of the differences in noradrenaline depletion.

Several authors have reported that noradrenaline disappears more slowly after reserpine in organs deprived of their sympathetic innervation (Karki, Paasonen and Vanhakartano 1959 Hertting, Potter and Axelrod 1962 Weiner Perkins and Sidman 1962, Benmiloud and Euler 1963 Sedvall 1964) In the present study decentralization did not affect the noradrenaline depleting rate after reserpine in the genital organs, while, on the other hand, the submaxillary glands seemed to react more in accordance with the previous findings for other organs This could perhaps be explained by a lower nerve impulse frequency in vi o in the nerves to the genital organs, which are supposed to function only intermittently

This investigation was supported by grants from Stiftelsen Magnus Bergvalls Minne (to N. S.) and from the Medical Faculty Karolinska Institutet (Reservationsanslaget, to G. S.) Reserpine (Serpasil®) was kindly put at our disposal by CIBA A.G Vallingby

References

BENMILOUD, M. and U. S. v. EULER, Effects of bretylium, reserpine, guanethidine and sympathetic denervation on the noradrenaline content of the rat submaxillary gland. *Acta physiol. scand.* 1963. *59* 34—42.

BERTLER, Å. Effect of reserpine on the storage of catechol amines in brain and other tissues. *Acta physiol. scand.* 1961 *51* 75—83.

CARLSSON, A. Drugs which block the storage of 5-hydroxytryptamine and related amines. In *Handbuch der Exp. Pharmakol.* (ed. V. Erspamer) Springer Verlag, Berlin-Göttingen-Heidelberg. 1965 *19* 529—592.

CARLSSON, A. and N. Å. HILLARP Release of adrenaline from the adrenal medulla of rabbits produced by reserpine. *Kgl. Fysiogr. Sällsk. Lund Förh.* 1956. 26. No. 8.

CARLSSON, A., E. ROSENGREN, Å. BERTLER and J. NILSSON, Effect of reserpine on the metabolism of catechol amines. In: *Psychotropic Drugs.* Eds. S. Garattini and V. Ghetti. Elsevier Amsterdam 1957 363—372.

EULER, U. S. v. and F. LISHAJKO, Improved technique for the flourimetric estimation of catecholamines. *Acta physiol. scand.* 1961 *51* 348—356.

EULER, U. S. v. and F. LISHAJKO A specific kind of noradrenaline granules in the vesicular gland and the vas deferens of the bull. *Life Sci.* 1966 *5* 687—691

FALCK, B., CH. OWMAN and N. O. SJÖSTRAND, Peripherally located adrenergic neurons innervating the vas deferens and the seminal vesicle of the guinea-pig. *Experientia* (Basel) 1965. *21* 98—103.

HERTTING, G., L. T. POTTER, and J. AXELROD, Effect of decentralization and ganglionic blocking agents on the spontaneous release of H³-norepinephrine *J. Pharmacol.* 1962. *136* 289—292.

HOLZBAUER, M. and M. VOGT Depression by reserpine of the noradrenaline concentration in the hypothalamus of the cat. *J. Neurochem.* 1956. *1* 8—11

KOPIN, I. J., E. K. GORDON and W. D. HORST Studies of uptake of 1-norepinephrine-C. *Biochem. pharmacol.* 1965. *14* 753—759

KÄRKI, N. T., M. K. PAASONEN and P. A. VANHAKARTANO, The influence of pentolonium, mecamylamine and yohimbine on the nordrenaline depleting action of reserpine. *Acta pharmacol.* (Kbh.) 1959 *16* 13—19.

NORBERG, K. A., Drug-induced change in monoamine levels in the sympathetic adrenergic ganglion cells and terminals. *Acta physiol. scand.* 1965. *65* 221—234

OHLIN, P. Nervous and hormonal control of salivary glands in rats. *Acta Univ. Lund* 1966. II 7

OWMAN, CH. and N. O. SJÖSTRAND, Short adrenergic neurons and catecholamine-containing cells in vas deferens and accessory male genital glands of different mammals. *Z. Zellf. Mikr.* 1965. 66. 300—320.

OWMAN, CH. and N. O. SJÖSTRAND, On short adrenergic neurons in the accessory male genital organs of the bull. *Experientia* (Basel) 1966 *22* 759—64.

SEDVALL, G., Short term effects of reserpine on noradrenaline levels on skeletal muscle. *Acta physiol. scand.* 1964. *62* 101—108.

SJÖSTRAND, N. O., Effect of reserpine and hypogastric denervation on the noradrenaline content of the vas deferens and the seminal vesicle of the guinea-pig. *Acta physiol. scand.* 1962 *56* 376—380

SJÖSTRAND, N. O. The adrenergic innervation of the vas deferens and the accessory male genital glands. *Acta physiol. scand.* 1965 *65* Suppl. 257

STJÄRNE, L. and F. LISHAJKO, Comparison of spontaneous loss of catecholamines and ATP in vitro from isolated bovine adrenomedullary, vesicular gland, vas deferens and splenic nerve granules. *J. Neurochem.* 1966 *13* 1213—1216

WURTMAN, R. M. PETERS and R. L. SIDMAN Effect of reserpine on noradrenaline content of innervated and denervated brown adipose tissue of the rat. *Nature* (Lond.) 1962 *193* 137—138.

In the present investigation the influence of barbiturate anesthesia and body temperature on the local 5-HT-edema was studied. 5-HT was used in different doses. Light anesthesia was used under both normothermia and hypothermia, and trypan blue was used in a dose which does not cause hemoconcentration in hypothermic mice.

Material and methods

Animals. 120 male albino mice, 4—7 months old and weighing 27—34 g were used.

5-hydroxytryptamine (5-HT) (Serotonine creatinine sulphate, Koch-Light Laboratories Ltd.) was used as 1 10 and 100 µg/ml solutions in isotonic saline, of which 0.04 ml was injected subcutaneously into the abdominal wall of the mice. Fresh solutions were prepared each morning.

Dye leakage. The local edema caused by 5-HT was estimated by the following technique 5 ml/kg b.w. of 1/2 % solution of trypan blue was given i. just prior to the injection of 5-HT The mice were killed 30 min later The local accumulation of trypan blue caused by 5-HT was determined by extraction and measurements in spectrophotometer (Judah and Willoughby 1962, Svanes 1965)

Anesthesia normothermia. The mice were anesthetized with pentobarbital and kept at normal body temperature. The last half hour of the experiment the anesthesia was light with active corneal reflex and respiratory frequencies between 120 and 160 per minute (Svanes 1967)

Hypothermia. The mice were anesthetized with pentobarbital and cooled to rectal temperature of 21—22 C, as described previously (Svanes 1967) During the last half hour of the experiment the anesthesia was light with the presence of the corneal reflex and respiratory frequencies between 30 and 50 per minute.

Analyses of variance were used in the statistical treatment of the results recorded in Table I

Results

The mice were distributed at random in 12 groups (Table I) Four groups of mice were not anesthetized. They were kept in a room at 24 C during the experiment. Four groups were anesthetized and kept at normal body temperature, and 4 groups were anesthetized and cooled to body temperature of 21—22° C. All mice were given an injection of trypan blue The injection was given after 1 hr of anesthesia to the anesthetized normothermic mice, and after 1½ hour of hypothermia to th hypothermic mice Immediately after the injection of trypan blue c. injection of 5-HT was given. The concentration of 5-HT which was used for the different groups, is shown in the table. The mice were killed 30 min after the injection of 5-HT The dye leakage was determined as described abov

The results are given in Table I. In unanesthetized mice 100 µg ml caused great dy leakage 10 µg ml caused a moderate dye leakage and 1 µg ml caused minimal or no dy leakage The anesthesia greatly reduced the dye leakage caused by 100 µg ml but did not modify notably the dye leakage caused by 10 and 1 µg ml. Analysis of variance disclosed a statistically significant effect of anesthesia ($P < 0.005$) and a significant interaction between anesthesia and concentration of 5-HT

TABLE I. Leakage of circulating trypan blue induced in mice by subcutaneous injection of 5-hydroxytryptamine.

Additional treatment	5-hydroxy tryptamine (μg/ml)	Number of mice	Extracted trypan blue	Dye leakage
None	100	10	0.111±0.056	0.091±0.056
	10	10	0.040±0.014	0.020±0.014
	1	10	0.020±0.005	0.000±0.005
	0	9	0.020±0.006	
Light anesthesia, normothermia	100	10	0.046±0.015	0.029±0.015
	10	9	0.034±0.015	0.017±0.015
	1	10	0.019±0.006	0.002±0.006
	0	9	0.017±0.005	
Light anesthesia, hypothermia	100	9	0.019±0.009	0.006±0.009
	10	10	0.024±0.011	0.011±0.011
	1	10	0.017±0.008	0.003±0.008
	0	10	0.013±0.006	

Extinction values. 0.067 E=5 μg. Mean ±S.D

Difference between trypan blue values of 5-hydroxytryptamine-treated mice and the mean value of the corresponding control mice. Mean ±S.D

<0.005) In the hypothermic mice 100 μg/ml and 10 μg/ml 5-HT caused less dye leakage than in the anesthetized normothermic mice, and 10 μg/ml caused greater dye leakage than did 100 μg/ml. A comparison of the hypothermic groups with the anesthetized normothermic groups disclosed a statistically significant effect of hypothermia (P<0.005) and a significant interaction between hypothermia and concentration of 5-HT (P<0.005).

Fig. 1 illustrates these different edema responses to HT in the three main groups of animals.

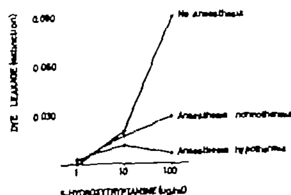


Fig. 1. Leakage of circulating trypan blue induced ... mic by subcutaneous injection of 5-hydroxytryptamine. The points represent mean values taken from table 1.

Discussion

The present investigation has shown that the edema provoking effect of s.c. injected 5-HT in mice is reduced by light anesthesia and even more by hypothermia, and the effect of anesthesia and hypothermia is highly dependent on the dose of 5-HT used. In an attempt to explain these findings the possible local actions of 5-HT must be discussed. Considerable evidence has been provided that 5-HT may act differently on different types of vessels, causing constriction of arteries, arterioles and veins, and dilatation, increased permeability and stasis in the venules (Haddy et al. 1957 Majno et al. 1961 Rowley 1964 Svanes 1968) The dilatation of venules caused by 5-HT indicates that the hydrostatic pressure in the venules was elevated. The mean capillary hydrostatic pressure is determined by the central arterial and venous pressures and by the ratio between pre and postcapillary resistance (Pappenheimer and Soto-Rivera 1948, Mellander 1960)

Probably 5-HT causes edema then by producing elevated hydrostatic pressure in the venules as well as increased permeability of the venular wall. Rowley (1964) provided evidence that the increased permeability might result from the elevated pressure in the venules. This pressure elevation could result from 5-HT reducing the precapillary to postcapillary resistance ratio. The degree of edema development on a certain 5-HT dose would be expected to be closely connected to its actual influence on this resistance ratio. In the presence of marked venous constriction (which was observed by Rowley) much edema could be expected if at the same time the arteries and arterioles were slightly constricted, and less edema if they were markedly constricted. If the relative effects of 5-HT on different types of vessels varies with the dose applied, this could perhaps explain some of the present findings.

Light pentobarbital anesthesia caused a great reduction in the local edema caused by 100 µg/ml 5-HT while the edema caused by 10 µg ml was not significantly influenced by the anesthesia. Pentobarbital anesthesia leads to increased response to administered 5-HT in mice (Svanes 1968) The local vascular response to injected 5-HT can therefore be expected to be increased under anesthesia The small edema formation after s.c. injection of 100 µg ml 5-HT in anesthetized mice could be explained if in this situation there was increased or prolonged constriction of arteries and arterioles.

The local edema caused by s.c. injection of 5-HT was in general less in hypothermic than in normothermic mice, and the edema caused by 100 µg ml 5-HT was relatively more reduced under hypothermia than that caused by 10 µg ml. Again this could be explained if at the larger dose the effect on arteries and arterioles was especially marked Under hypothermia the concentration of 5-HT in biophase will probably remain at a high level for a longer period of time than in normothermia because of delayed enzymatic inactivation, slowed diffusion and resorption, and reduced edema formation Consequently a prolonged action of 5-HT must be expected under hypothermia, as has been found for injected 5-HT in mice (Svanes

1968) The scanty edema caused by great doses of 5-HT under hypothermia may therefore well in part be due to increased or prolonged constriction of arteries and arterioles. However at a body temperature of 21 C the arterial pressure is probably reduced (Brendel, Albers and Usinger 1958 Bullard 1959) and the viscosity of blood is increased (Bullard 1959 Burton 1960) and both these factors contribute to reduce the edema formation under hypothermia.

The present investigation was supported by grants from L. Meltzer' Høyskolefond and Nils Henrichsens og hustru Anna Henrichsens legat.

References

Brendel, W C Albers, and W Usinger, Der Kreislauf in Hypothermie (Die einzelnen Kreislaufgrössen und ihre Koordination während der Auskühlung) *Pflügers A. k. P Physiol.* 1958. 266. 341—356.

Bullard, R. W Cardiac output of the hypothermic rat. *Amer J Physiol.* 1959 196 415-419

Burton A. C Hemodynamics and the physics of the circulation. Pp. 643—666, in Ruch T C. and Fulton, J F Medical physiology and biophysics. Ed. 18, Philadelphia, W. Saunders Co 1960 1232 pp.

Haddy F J M Fleishman and D A. Emanuel, Effect of epinephrine norepinephrine and serotonin upon systematic small and large vessel resistance. *Circ. Res.* 195 5 247-251

Judah, J D and D A. Willoughby A quantitative method for the study of capillary permeability Extraction and determination of trypan blue in tissues. *J Path. Bact* 196 83 (2) 567—572.

Majno G G E Palade and G I Schoefl, Studies on Inflammation II The site of action of histamine and serotonin along the vascular tree A topographic study *J biophys biochem. Cytol.* 1961 11 607—626.

...lander, S Comparative studies on the adrenergic neurohumoral control of resistance and capacitance blood vessels in the cat. *Acta physiol. scand.* 1960. 50 Suppl. 176. pp 1—86

...appenheimer, J R and A. Soto-Rivera, Effective osmotic pressure of the plasma proteins and other quantities associated with the capillary circulation in the hindlimbs of cats and dogs *Amer J Physiol.* 1948. 152 471—491

Rowley D A Venous constriction as the cause of increased vascular permeability produced by 5-hydroxytryptamine, histamine, bradykinin and 48/80 in the rat. *Brit J ... Path* 1964 45 56—67

Svanes K Studies in hypothermia 3 The rôle of reduced release of chemical mediators in turpentine inflammation in hypothermic mice. *Acta path. microbiol. sc... d* 1965 65 15—23

Svanes K Studies in hypothermia 8 Relation between depth of pentobarbital anesthesia, capillary circulation and formation of turpentine edema, under hypothermia and normothermia *Acta ... th. scand* 1967 11 1—13

Svanes, K., Microcirculatory changes and general responses to 5-hydroxytryptamine in normothermic and hypothermic mice *Acta physiol. sc... d.* 1968. 72 (In press)

Acta physiol. scand. 1968 72. 383—384

From the Department of Physiology, Gymnastik- och Idrottshögskolan, and the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

Muscle Tissue Lactate after Maximal Exercise in Man

By

BERTIL DIAMANT, JAN KARLSSON and BENGT SALTIN

Blood lactate determinations have been routine in connection with exercise studies, but so far muscle tissue lactate levels on humans have not been demonstrated.

Four physical education students were studied at rest and within 5 sec immediately after an extremely heavy exercise load (bicycle ergometer) sufficient to exhaust the subjects in 3 min. By using the muscle biopsy technique described by Bergström (1962) muscle tissue samples (15—30 mg wet weight) were obtained from the thigh muscles (M. quadriceps femoris). To determine lactate concentration in the muscle sample a modification of the fluorimetric method described by Lowry (1964) was used. After the muscle specimen was obtained the following main steps were followed.

1. Immediate freezing of the sample in liquid nitrogen, and thereafter the sample was kept at temperatur below −80 C.
2. Weighing of the powdered sample at −20 C.
3. deproteinizing the sample by placing it on the top of 0.1—0.2 ml of frozen 3 M HClO and then thawed at +4 C.
4. the NAD-linked reaction was performed in 0.1 M carbonate buffer. Lowry (1964) uses bovin serum albumin in this buffer but we have not, in order to minimize the risks for contamination with the lactate. The blood lactate concentration was determined on arterialized fingertip blood using an enzymatic method (Scholz *et al.* 1959)

The resting muscle tissue lactate level was in 4 subjects somewhat higher than in the blood, with a mean of 3.0 mM in the muscle (wet weight) and 1.4 mM in the blood (Table I). After maximal exercise the mean muscle tissue lactate concentration was 19.1 compared to 11.4 mM in the blood.

TABLE I. Lactate concentrations at rest and after maximal exercise in blood and muscle tissue

Subject	Rest		Maximal exercise	
	muscle	blood	muscle	blood
I	2.7	1.5	11.2	7.3
II	2.3	1.3	19.0	11.2
III	1.8	1.7	24.6	14.5
IV	3.2	1.2	21.6	12.4
Mean	3.0	1.4	19.1	11.4

m moles per kg wet muscle
m moles per l

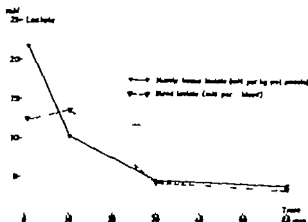


Fig. 1 Muscle tissue (m. quadriceps femoris) and blood lactate concentration in one subject immediately, 10 30 and 60 min after maximal bicycle exercise.

In one subject (IV) muscle samples and blood samples were also obtained, 10 30 and 60 min after the maximal exercise (Fig 1) The reduction of the muscle tissue lactate concentration after maximal exercise seems to be asymptotically approaching resting values whereas the blood concentration shows the typical pattern with a peak value around 10 min after exercise (*cf* Åstrand 1960 Bang 1936, Margaria *et al.* 1963 and Scholz *et al.* 1959)

Muscle tissue and blood lactate values immediately after exercise on per liter water basis, were 12.5 mM for blood and 24.5 mM for muscle tissue. The intracellular ...tion of lactate in the muscle may be higher as the muscle sampl according ...um (1962) contains 20 per cent extracellular fluid. Considering this, there ...d gradient between the muscle and the blood, which is most pronounced immediately after exercise but also present at rest.

Supported by the Swedish Medical Research Council project No K 67 14X 2203-01

References

ÅSTRAND, I Aerobic work capacity in men and women. *Act physiol* *d* 1960 *49* Suppl. 169 14—16.

BANG, O The lactate content of the blood during and after muscular exercise man. *Ska d. Arch. Physiol.* 1936 *74* Suppl. 10.

BERGSTRÖM J Muscle electrolytes in man. *S and J h Lab Invest* 1962 Suppl. 68.

LOWRY O H. and J V PASSONNEAU The relationships between substrates and enzymes of glycolysis in brain. *J biol Chem.* 1964 *239* 31—42

MARGARIA, R., P CERRETELLI P E DI PRAMPERO C MASSARI and G TORELLI Kinetics and mechanisms of oxygen debt contraction in man *J ppl Physiol* 1963 *18* 371—377

SCHOLZ, R. H. SCHMITZ, T B CHER and J O LAMPE Übe die Wirkung on ... auf Bäckerhefe. *Bioch J* 1959 *71* 331 71—86

Acta physiol. scand. 1968. 72. 385—391

From the Department of Physiology, Karolinska Institutet, Stockholm, Sweden

Inhibition by Phenoxybenzamine of the Noradrenaline Releasing Effect of Tyramine

By

P. HEDQVIST, A. OLIVERIO and L. STJÄRNE

Received 18 February 1967

Abstract

HEDQVIST, P., A. OLIVERIO and L. STJÄRNE, *Inhibition by phenoxybenzamine of the noradrenaline releasing effect of tyramine.* Acta physiol. scand. 1968. 72. 385—391.

After loading the isolated, perfused dog spleen with tritium-labeled noradrenaline, intra-arterial injections of tyramine were found to cause an increase in the overflow of both labeled, exogenous, and of endogenous noradrenaline into the effluent from the organ. The overflow response to 2—3 consecutive injections of a standard dose of tyramine remained relatively constant. Increasing the tyramine dose up to 100-fold resulted in a marked [illegible] in the pressor response but did not produce a proportional increase in the overflow of noradrenaline. Phenoxybenzamine added to the perfusion fluid strongly reduced or completely abolished the overflow response to tyramine.

The sympathomimetic effect of tyramine (TA) has been shown to be accompanied by increased efflux of noradrenaline (NA) from sympathetically innervated [illegible] (Lockett and Eakins 1960, Lindmar and Muscholl 1961, Stjärne 1961 and [illegible]). The direct NA-liberating effect of TA appears to be further magnified by [illegible] of NA rebinding in the axon (Iversen 1964, Lindmar and Muscholl 1[illegible]).

In the course of a study of the mechanism of the release of NA caused by TA, it was observed that it was strongly inhibited or completely abolished by phenoxybenzamine (PBA).

Material and methods

30 mongrel puppies were used for the investigation. After anesthetization [illegible] tion of a mixture of sodium pentobarbital [illegible] mg/kg and urethane [illegible] domen was opened by a midline incision and the splenic artery [illegible] ected free. The spleen was then excised and transferred to [illegible] vascular system was immediately flushed with a solution containing [illegible] saline and then connected to a constant rate perfusion pump (Sigma [illegible]) flow rate of about 10 ml/min. A modified Tyrode solution [illegible]

Abbreviations: TA = Tyramine, NA = Noradrenaline, PBA = Phenoxybenzamine.

per cent dextran (Macrodex® M_w 70 000) and aerated with 5 per cent carbon dioxide in oxygen, was used as perfusion medium. The temperature was maintained at 37 C. bic acid (20 µ/ml) was added to stabilize the NA. The effluent was divided into 10 ml fracti an automatic fraction collector Hydrochloric acid (1N) was infused into the effluen the organ, t constant rate, to maintain a suitable acidity for the protection of NA.

40 µC of H-dl-noradrenaline (New England Nuclear Corp., specific acti ity 5—7 C/m... chromatographically purified adn stored at —30 C in 1 % solution of sodium metabisulphite) was infused i.a. for 15 min. After an interval of 30 min the spleen was exposed alternatingly to supramaximal nerv stimulation and i.a. TA injection. The nerv stimulation was carried out by means of Grass S 4 stimulator using shielded platinum electrodes (10 V 10/sec, duration 10 msec, stimulation periods 20—60 sec) After an interval of 15 min TA was injected i.a. in amounts from 10—1 000 µg. PBA was then in some experiments added t the perfusion medium at a concentration of 10 µg/ml and the electrical nerve stimulations and TA injections were repeated.

The NA content of the perfusate fractions was determined fluorimetrically after purification on aluminum oxide. 0.5 ml aliquots of the perfusate were added to als with 20 ml of a 7 3 toluene-absolut ethanol mixture containing 4 g of 2 5-diphenyloxazole and 100 mg of 1-4-bis-2(4-methyl-5-phenyloxazolyl) benzene per liter of toluene, and counted for 10 min in a Packard Liquid Scintillation Spectrometer Quenching was monitored by internal standards.

In some cases the perfusate was analyzed by cation exchange olumn chromatography on Amberlit CG 120 according to the method of Stjarne and Lishajko (1967)

Results

In the absence of drug treatment i.a. injections of TA into the isolated dog spleen at a dose of 10 µg consistently produced a small pressor response and increased the amounts of both labeled and fluorimetrically determined NA in the enous effluent (Fig 1 2) Both the pressor and the overflo responses remained largely constant, or decreased only moderately on 2—3 consecutive injections of 10 µg of TA. However while raising the TA dose up to 100-fold markedly increased the pressor

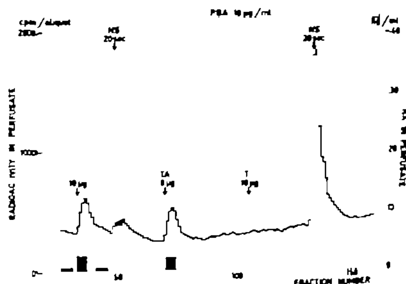


Fig. 1 Overflow of radioacti material and of fluorimetrically determined NA in response to nerv stimulation and to TA injection Effect of PBA. Filled columns: NA in perfusate (fluorimetrically determined)

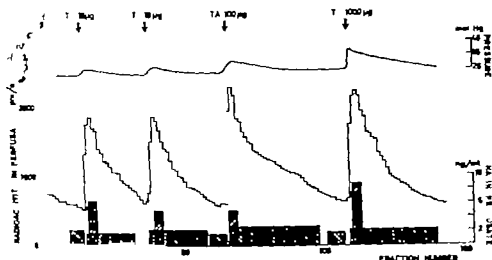


Fig 2. Changes in perfusion pressure, and overflow of radioactive material and of fluorimetrically determined NA in response to TA injections. Effect of increasing doses of TA.

response, it did not alter the peak level of the NA overflow response to any considerable extent, although often prolonging its duration (Fig 2). Thus at all dose levels used in the experiments (10—1 000 µg) TA caused only a 2—4 fold increase in the labeled (Table I) and fluorimetrically determined NA overflow from the spleen.

Electrical nerve stimulation at a frequency of 10/sec for 20 sec resulted in a much stronger increase in the peak level overflow of both endogenous and labeled NA into the perfusate than did the injections of TA (Fig 1).

Addition of PBA (10 µg/ml) to the perfusion fluid strongly potentiated the overflow response to nerve stimulation, while the labeled NA overflow response to TA was completely abolished, and that of endogenous NA strongly reduced (Fig 1, cf Table I).

Analysis of the perfusate by cation exchange column chromatography revealed that most of the radioactivity found after nerve stimulation (more than 80 per cent) or after TA (more than 70 per cent) consisted of intact NA. During the resting periods intact NA accounted for only a small proportion of the total radioactivity in the perfusate (less than 10 per cent) (Fig 3).

TABLE I. Per cent increase of radioactivity in the perfusate from isolated dog spleen in response to i.a. injections of different amounts of TA. Means ± SEM

	10 µg	100 µg	1000 µg
Control	203 ± 23, n = 12	210 ± 32, n = 4	204 ± 28, n = 4
PBA	—0.8 ± 3.1, n = 7		

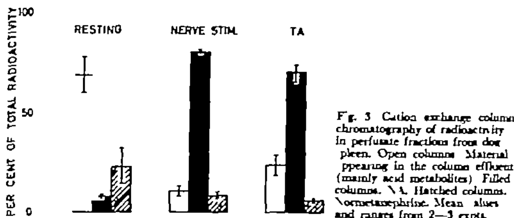


Fig. 3 Cation exchange column chromatography of radioactivity in perfusate fractions from dog spleen. Open columns Material appearing in the column effluent (mainly acid metabolites) Filled columns. NA. Hatched columns. Normetanephrine. Mean values and ranges from 2—3 expts.

Discussion

In the present experiments the NA overflow response to TA showed the following features. It remained largely unchanged on 2—3 consecutive i.a. injections of a standard dose of TA. Yet increasing the TA dose up to 100-fold, which resulted in a considerable rise in the amplitude of the pressor response, caused no or only a very modest increase in the peak level and only a less than twofold increase in the total NA overflow Finally the overflow response to TA was strongly inhibited by PBA.

The absence of rapid tachyphylaxis in the NA overflow response observed in the present experiments appears to be in agreement with the data published from similar experiments with the isolated, perfused rat heart (Axelrod *et al* 1962)

The observed near-absence of a dose response relationship in the peak level of NA overflow caused by TA is in general agreement with previous findings of very flat dose-response curve for the NA overflow caused by TA in the rabbit heart (Lindmar and Muscholl 1961 It probably indicates that even the lowest dose of TA used in the present experiments was "supramaximal" i e exceeded the level required to produce the maximum level of NA overflow possible in response to a single injection of TA. However since octopamine (OA) formed from the injected challenging doses of TA (Musacchio and Goldstein 1963 Chidsey Kaiser and Lehr 1964 Carlsson and Waldeck 1963 might to some extent have replaced NA in the preparation, the possibility should not be overlooked that determination of NA release after previous injection of relatively large amounts of TA might give an underestimate of the total amine (NA plus OA) release

The rise in the amplitude of the pressor response to increasing doses of TA, occurring in spite of a largely unchanged NA overflow suggests that the mechanical response might in part be due to a smooth muscle stimulating effect of TA (Eliasson and Astrom 1955) However the constancy in NA overflow to increasing doses of TA may not accurately mirror the magnitude of the actual NA release from the neuron, and thus the local concentration of free extra axonal NA, since the in-

nerve vasoconstriction in itself would tend to promote reuptake of NA released by TA (Rosell, Kopin and Axelrod 1963) while the increase in the local concentration of TA would have the opposite effect, inhibiting reuptake of NA (Lindmar and Muscholl 1965). Thus the degree of participation of NA released by TA in producing the mechanical response remains difficult to judge.

The finding that PBA, at a dose which caused the expected potentiation of the NA overflow response to nerve stimulation (*cf* Brown and Gillespie 1957) strongly reduced the endogenous and completely abolished the labeled NA overflow response to TA is in agreement with the observations of Benfey and Greeff (1961) and Swaine (1965) but in contrast to other reports, according to which PBA does not affect (Gutman and Weil-Malherbe 1966) or potentiates (Werner Draskoczy and Burack 1962) the NA releasing effect of TA.

While there is general agreement that TA exerts its sympathomimetic effect by raising the concentration of NA extraneuronally both by increasing the efflux of NA from the axon and by preventing NA recapture into the axon (*cf* references under introduction) the exact locus from which NA is released by TA has not been defined (*cf* I ersen 1967). However on the basis of studies of the inhibition of the sympathomimetic effect of TA by cocaine and similar drugs the concept has been proposed that initial transport of TA into the interior of the axon is a prerequisite for its NA releasing effect (Furchgott *et al.* 1963). This concept is supported by the finding in the present study that PBA blocks the TA induced NA release, since PBA has been found to exert a cocaine like inhibitory effect on the amine "pump" in the axonal membrane transporting NA (Thoenen, Hürlimann and Haefely 1963 Andén *et al.* 1964) and therefore presumably also TA into the neuron. The capacity of this highly efficient "pump" which in the spleen removes about 70 per cent of a moderate NA load from the perfusion medium during one single passage (Thoenen *et al.* 1965 Gillespie and Kirpekar 1965) appears to be dimensioned according to the physiological range of NA loads which can occur in the extracellular fluid as a result of nerve activity and of secretion from chromaffin tissue into the circulation (*cf* Gillespie *et al.* 1966). Thus it seems likely that even the lowest dose of TA (10 μg i.a.) injected in the present experiments might be supramaximal i.e. saturate the axonal membrane amine "pump". The limited capacity of the mechanism transferring TA from the circulation to the interior of the axon would then be the cause of the near-absence of a dose response relationship in the peak level of the NA overflow response to TA.

Apparently TA exerts its NA mobilizing effect on targets located inside the neuron. According to recent evidence TA accelerates the spontaneous loss of NA from isolated storage particles from nerve tissue (Euler and Lishajko 1960) both by a direct NA liberating effect (Euler and Lishajko 1968) and by inhibiting reuptake of the NA released (Stjärne 1966, Euler and Lishajko 1968). In fact the effect of TA at the storage particle level shows several features which might be relevant to the present study. Thus the NA liberating effect of TA at this level does not increase linearly with the TA dose throughout the TA concentration range (Euler and

Luhajko 1960) Moreover the effect of TA is inhibited by PBA, although admittedly at a relatively high concentration (Euler and Lishajko 1968)

Whether the "TA releasable pool" of NA defined on the basis of pharmacolog evidence obtained *in vivo* (Potter Axelrod and Kopin 1962) corresponds to the particle-bound NA studied in the above mentioned *in vitro* experiments cannot be positively established at the present time. However the "TA releasable pool of NA has been negatively defined as being different from that from which NA is released by nerve stimulation (Axelrod *et al.* 1962) This view is supported by the observation that the specific activity of the NA overflowing from perfused tissues in response to TA was more closely related to that overflowing during the resting period than during nerve stimulation (Chidsey and Harrison 1963) That observation suggests that the mechanism of the TA-induced increase in NA output from the neuron may differ from that of electrical depolarisation in representing acceleration of the process of NA leakage, probably from the entire neuron, occurring even under resting conditions, rather than active discharge from its specialized synaptic parts. The primary mechanism of action of the TA transported into the neuron might thus be active liberation of bound NA (Euler and Lishajko 1968) and the second mechanism intraaxonal inhibition of rebinding of NA. Both processes would result in a net increase in free i.e. diffusible, NA in the axon, protected from deamination by simultaneous competitive inhibition of monoamine oxidase for which TA is a much better substrate than NA (*cf* Kopin 1964) The rise in the diffusion gradient for NA would result in accelerated leakage and concomitant block by TA of reuptake of NA by the axonal membrane amine pump would produce the increased overflow essentially intact NA observed in the present experiments (*cf* Kopin and Gordon)

Thus in conclusion, the PBA induced block of the NA release normally occurring after TA supports the view that mutual transport of TA into the interior of the axon is a prerequisite for its NA releasing effect. The limitation of the capacity of the TA transporting system might be the basis for the observed absence of a dose-effect relationship in the NA release caused by TA On the basis of *in vit* evidence it appears conceivable that part of the PBA effect might be due to direct inhibition of the NA releasing effect of TA at the intraaxonal storage particle level However the exact nature of the "TA releasable NA pool" still remains unknown.

This study was supported by grants from the Swedish Medical Research Council, projects No. 14X 97-02, and 14X-625-01 as ll as from Magnus Bergvalls Stiftelse and from Stift ben Lars Hiertas Minne Each ar be eby grateful lly acknowledged

References

Axelrod, N E., H. Corrodi, M. Ettles, E. G. ... and H. ... Selective ... of some catecholamines by the isolated heart and its inhibition by cocaine and phenoxybenzamine. *Int pharmacol.* (*Abk*) 1964 21 247—259

Axelrod, J E. Gordon G Hertting, I J Kopin and L. T. Potter, On the mechanism of tachyphylaxis to tyramine in the isolated rat heart *Brit J Pharmacol.* 1962 19 56—63

Benfey, B. G. and K. Greeff, Interactions of sympathomimetic drugs and their antagonists on the isolated atrium. Brit. J. Pharmacol. 1961. 17 232—235.

Brown, G. L. and J. S. Gillespie, The output of sympathetic transmitter from the spleen of the cat. J. Physiol. (Lond.) 1957 138 81—102.

Carlsson, A. and B. Waldeck, β-Hydroxylation of tyramine in vivo. Acta pharmacol. (Kbh.) 1963. 20 371—374.

Chidsey, C. A. and D. C. Harrison, Studies on the distribution of exogenous norepinephrine in the sympathetic neurotransmitter store. J. Pharmacol. exp. Ther. 1963. 140 217—223.

Chidsey, C. A., G. A. Kaiser and B. Lehr, The hydroxylation of tyramine in the isolated canine heart. J. Pharmacol. exp. Ther. 1964. 144 393—398.

Eliasson, R. and A. Åström, Pharmacological studies on the perfused human placenta. Acta pharmacol. (Kbh.) 1955. 11 254—264.

Euler, U. S. v. and F. Lishajko, Release of noradrenaline from adrenergic transmitter granules by the action of tyramine. Experientia 1960. 16 376—377.

Euler, U. S. v. and F. Lishajko, Effect of directly and indirectly acting sympathomimetic amines on adrenergic transmitter granules. Acta physiol. scand. 1968. 73 In press.

Furchgott, R. F., S. M. Kirpekar, M. Rieker and A. Schwab, Actions and interactions of norepinephrine, tyramine and cocaine on aortic strips of rabbit and left atria of guinea pig and cat. J. Pharmacol. exp. Ther. 1963. 142 39—58.

Gillespie, J. S. and S. M. Kirpekar, The inactivation of infused noradrenaline by the cat spleen. J. Physiol. (Lond.) 1965. 176 205—227.

Gillespie, J. S., D. N. H. Hamilton, R. J. A. Hosie, R. F. Macadam and H. McCaffery, Histological and subcellular localization of noradrenaline infused into the cat spleen. J. Physiol. (Lond.) 1966. 187 4—5 P.

Gutman, Y. and H. Weil-Malherbe, Kinetics of catecholamine release by tyramine. Life Sci. 1966. 5. 1293—1298.

Iversen, L. L. The inhibition of noradrenaline uptake by sympathomimetic amines. J. Pharm. Pharmacol. 1964. 16 435—437.

Iversen, L. L., The uptake and storage of noradrenaline in sympathetic nerves. Cambridge Univ. Press. 1967.

Kopin, I. J. Storage and metabolism of catecholamine. The role of monoamine oxidase. Pharmacol. Rev. 1964 16 179—191.

Kopin, I. J. and E. K. Gordon, Metabolism of administered and drug-released norepinephrine-7-H^3 in the rat. J. Pharmacol. exp. Ther. 1963 140 207—216.

Lindmar, R. and E. Muscholl, Die Freisetzung von Noradrenalin aus dem perfundierten Kaninchenherzen. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 1961 241 528.

Lindmar, R. and E. Muscholl, Die Verstärkung der Noradrenalinwirkung durch Tyramin. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 1965. 252 122—133.

Lockett, M. F. and K. E. Eakins, Chromatographic studies of the effect of intravenous injections of tyramine on the concentration of adrenaline and noradrenaline in plasma. J. Pharm. Pharmacol. 1960. 12. 513—517.

McEwen, L. M. The effect on the isolated rabbit heart of vagal stimulation and its modification by cocaine, hexamethonium and ouabaine. J. Physiol. (Lond.) 1956 131 678—689.

Musacchio, J. M. and M. Goldstein, Biosynthesis of norepinephrine and norsynephrine in the perfused rabbit heart. Biochem. Pharmacol. 1963 12 1061—1063.

Potter, L. T., J. Axelrod and I. J. Kopin, Differential binding and release of norepinephrine and tachyphylaxis. Biochem. Pharmacol. 1962. 11 254—256.

Rosell, S., I. J. Kopin and J. Axelrod, Fate of H^3-noradrenaline in skeletal muscle before and following sympathetic stimulation. Amer. J. Physiol. 1963. 205 317—321.

Stjärne, L., Tyramine effects on catecholamine release from spleen and adrenals in the cat. Acta physiol. scand. 1961 51 224—229.

Stjärne, L. Storage particles in adrenergic tissues. Pharmacol. Rev. 1966 18 425—432.

Stjärne, L. and F. Lishajko, Localization of different steps in noradrenaline synthesis to different fractions of a bovine splenic nerve homogenate. Biochem. Pharmacol. 1967 16 1719—1728.

Swaine, C. R. Effect of adrenergic blocking agents on tyramine-induced release of catecholamines from the cat heart. Proc. Soc. exp. Biol. (N.Y.) 1963 112 388—390.

Thoenen, H., A. Hürlimann and W. Haefely, Removal of infused norepinephrine by cat spleen: mechanism of its inhibition by phenoxybenzamine. Experientia (Basel) 1963 19 601—602.

Weiner, N., P. R. Draskóczy and W. R. Burack, The ability of tyramine to liberate catecholamines in vivo. J. Pharmacol. exp. Ther. 1962 137 47—55.

Acta physiol. scand. 1968, 72, 392—395

From the Department of Pharmacology, University of Göteborg, Sweden

Stereospecificity and Intracellular Binding of Metaraminol

By

PER LUNDBORG and ROBERT STITZEL

Received 19 June 1967

Abstract

LUNDBORG, P. and R. E. STITZEL. *Stereospecificity and intracellular binding of metaraminol.* Acta physiol. scand. 1968. 72. 392—395.

Both *d*- and *l*-metaraminol (MA) were able to displace ³H-*dl*-MA from subcellular fractions of the mouse heart when given 15 min after administration of the labelled amine. The *l*-form was more effective, however. If the unlabelled isomers were given 24 hrs after ³H-*dl*-MA only the *l*-isomer was an effective displacing agent although it was less effective than when it was given 15 min after the tritiated racemic mixture. These results suggest that *d*-MA is not firmly bound to the particulate fraction and is rapidly lost from sympathetically innervated tissues. It is further suggested that there is a transfer of MA from more to less available granular storage sites with time.

Histochemical evidence indicates that the uptake and accumulation of injected metaraminol (MA) occurs within the adrenergic neuron (Jonsson and Ritzén 1966). MA is rapidly taken up and retained in sympathetic nerve endings (Shore, Busfield and Alpers 1964, Andén 1964, Gram and Wright 1966) and apparently can lower the catecholamine content of the rat heart by a mole for mole displacement of noradrenaline from its storage sites (Shore, Busfield and Alpers 1964, Andén 1964). Studies on the subcellular distribution of MA have demonstrated that this amine is stored, in part, by a particulate fraction derived from homogenates of sympathetically innervated tissue (Giachetti and Shore 1965, Lundborg and Waldeck 1966). It is quite probable, therefore, that MA is stored in noradrenaline binding sites. Shore and co-workers (1964) have presented evidence that *l*-MA, but not *d*-MA, is the active noradrenaline-depleting agent and that *l*- but not *d*-MA is taken up and retained in cardiac tissue. The above observations have prompted us to examine further the influence of stereospecificity on the binding of MA to subcellular fractions.

Methods

Mice, divided into groups of six, were given 40 µg/kg of ³H-*dl*-MA (100 mC/mM) intravenously. Control groups received no further treatment and were killed either 30 min or 24 hrs later. Other groups were injected with 0.25 mg/kg of either *d*- or *l*-MA (un-

TABLE I. Influence of *d* and *l* metaraminol (MA) on the H-*dl*-MA content of subcellular fractions of the mouse heart

Treatment	Number of expts.	H-MA ng/g Particulate	Supernatant	$\frac{P}{P+S}$
none	4	9.14±0.59	37.08±1.71	19.8±1.0
d-MA	4	6.21±0.38*	21.14±0.97*	22.7±1.0
l-MA	4	0.94±0.20*	8.46±0.47	9.8±1.4

Control animals received H-*dl*-MA (40 μg/kg L) alone. Unlabelled *d*- or *l* MA (0.25 mg/kg L) as given 15 min after H-*dl*-MA and all animals were sacrificed 30 min after receiving the labelled amine. The alues given in the table are means ± S.E.M. Each experiment as performed on six pooled hearts.
P<.001

labelled) 15 min or 24 hrs after the injection of the tritiated amine. All animals were killed 15 min after the injection of the unlabelled amine. Hearts were removed and homogenized in the cold using plastic pestle. A coarse fraction was obtained by centrifugation at 4 C at 2000 × g for 10 min. The resulting supernatant was then centrifuged at 100,000 × g for 60 min in Spinco Model L Ultracentrifuge, providing two more fractions, particulate and high speed supernatant. After protein precipitation the various fractions were placed on an ion exchange column (Dowex 50 W X4) and analyzed as described previously (Stitzel and Lundborg 1967)

Results

Approximately 30 per cent of the retained ^{3}H-*dl*-MA as recovered from the coarse fraction of heart homogenates. This agrees quite well with the distribution studies of Giachetti and Shore (1965) who, using unlabelled MA, demonstrated approximately 25 per cent of the amine in the coarse fraction. Since the coarse fraction contains several elements (nuclei, unbroken cells, membrane fragments, etc.) changes in its amine content are difficult to interpret and therefore like the bove authors, w ha e more closely followed the alterations in the particulate and supernatant fractions.

Table I illustrates the effect of unlabelled *d* and *l*-MA on the subcellular distribution and concentration of H-*dl*-MA given 15 min earlier. These data show that at this interval *d* MA can displace the labelled amine from both the supernatant and the particulate fractions. Twenty-four hours after the administration f ^{3}H-*dl*-MA, however the *d* isomer was irtually ineffective in displacing the labelled amine f om any subcellular site (Table II). The relati amounts of H-*dl*-MA found in the particulate and supernatant fractions $\left(\frac{P}{P+S}\right)$ at this time period were almost identical w th those of the control animals (Table II).

l MA, on the other hand, was able to displace ^{3}H-*dl* MA from both the particulate and supernatant fractions both 30 min and 24 hrs after the injection of the radio-

TABLE II. Influence of *d* and *l* metaraminol (MA) on the ^{3}H-*dl*-MA content of subcellular fractions of the mouse heart

Treatment	Number of expts.	^{3}H-MA-ng/g		$\frac{P}{P+S}$
		Particulate	Supernatant	
None	5	5.97 ± 0.61	22.95 ± 1.22	20.8 ± 2.4
d-MA	8	5.30 ± 0.32	22.70 ± 1.17	19.1 ± 1.3
l-MA	8	2.72 ± 0.28*	12.08 ± 0.61	18.1 ± 0.9

Control animals recei ed ^{3}H-*dl*-MA (40 μg/kg, i.) alone. Unlabelled *d*- or *l*-MA (0.25 mg/kg, i.) was given 23 3/4 hrs after ^{3}H-*dl*-MA and all animals were sacrificed 24 hrs after receiving the labelled amine. The values given in the table are means ± S.E.M. Each experiment was performed on six pooled hearts.
* $P < .001$

active compound (Table I and II) Even at the shorter interval *l*-MA was much more effective in displacing ^{3}H-*dl* MA than was the *d* isomer

A comparison of the actions of *l*-MA on the particulate fraction 15 min and 24 hrs after the administration of ^{3}H-*dl* MA indicates that the unlabelled amine was less effective at the longer time interval. Whereas after 30 min *l* MA could displace 90 per cent of the particle-bound ^{3}H-*dl* MA, only 55 per cent could be removed 24 hrs after injection of the labelled amine.

Discussion

The optical specificity of MA binding appears to be quite marked. Studies on the rate of disappearance of *d* and *l* MA indicate that while the *l* isomer is retained for many days *d* MA is essentially gone 3 hrs after i.v. administration (Shore, Busfield and Alpers 1964)

Our studies suggest that 15 min after the injection of ^{3}H-*dl* MA both isomers are found in the particulate and supernatant fractions. This is indicated by the ability of the unlabelled isomers to displace the labelled racemic mixture from both fractions. Although both *d* and *l* MA could displace ^{3}H-*dl* MA from subcellular fractions, *l* MA was the more effective displacing agent. These studies are interpreted to indicate that shortly after the injection of ^{3}H-*dl* MA both isomers are taken up into subcellular fractions of the mouse heart but they probably differ in their affinity for binding sites and/or uptake mechanisms. In this regard Lundborg (1966) has shown that the uptake of MA by adrenal granules favours the *l*-form.

24 hrs after the administration of ^{3}H-*dl* MA, however the unlabelled *d*-isomer was virtually incapable of displacing the labelled amine from any subcellular site, while the *l* form was still highly effective In the light of the studies of Shore *et al.* (1964) it is quite probable that little if any of the tritiated MA remaining in the

heart at this time is of the *d* form. Thus there is probably no ^{3}H-*d* MA available for exchange with the unlabelled isomer and the ^{3}H-*l* MA remaining is apparently not in a site readily accessible to the *d* form. Several workers have proposed the existence of a gradual transfer of MA from one subcellular storage site to another with time (Crout *et al* 1964 Lundborg and Stitzel 1967a) The present finding that particle-bound ^{3}H-*dl* MA could be more easily displaced shortly after its administration than after longer intervals is in agreement with such a proposal. Further support is given by our observation that *l* MA, although an effective displacing agent at both long and short intervals after ^{3}H-*dl* MA administration, was less effective at the longer interval. Furthermore, we have reported earlier (Lundborg and Stitzel 1967 b) that protriptyline was an effective MA-depleting agent only when MA was given shortly before the injection of protriptyline. All the above observations point to a transfer of MA from a more to a less readily available storage form.

Our studies and those of Giachetti and Shore (1965) support the assumption that both isomers of MA can be taken up by subcellular fractions of the heart, but that the *d*-isomer leaves the tissues at a faster rate. One reason for the relatively rapid loss of *d* MA may be that *d* but not *l* MA, forms only a loose and readily reversible association with adrenergic storage granules. The above results emphasize an inherent stereospecificity in the uptake and binding of MA.

The research reported in this manuscript has been supported by the Swedish State Medical Research Council (B 67 14X 155-03 A) and the Faculty of Medicine, University of Göteborg, Sweden. RES would like to thank West Virginia University for the leave of absence which allowed him to carry out this work, and the SSMRC for their financial support.
The competent technical assistance of Miss Lena Ramstedt is gratefully acknowledged

References

Andén, N. E., On the mechanism of noradrenaline depletion by α-methyl-meta-tyrosine and metaraminol. *Acta pharmacol.* (Kbh.) 1964 *21* 260—271
Crout J. R., H. S. Alpers, E. L. Tatum and P. A. Shore, Release of metaraminol (aramine) from the heart by sympathetic nerve stimulation. *Science* 1964. *145* 828—829.
Giachetti, A. and P. A. Shore, Intracellular distribution of metaraminol and related compounds in rat heart. *Life Sci.* 1965. *4* 1455—1460.
Gram, T. E. and H. Wagner Some factors influencing the action of metaraminol in rabbits *Arch. int Pharmacodyn.* 1966. *160* 294—311
Jonsson, G and M. Ritzén, Microspectrofluorometric identification of metaraminol in sympathetic adrenergic neurons *Acta physiol scand* 1966. *67* 505—513
Lundborg, P Uptake of metaraminol by the adrenal medullary granules *Acta physiol scand.* 1966 *67* 423—429
Lundborg, P and R. E Stitzel, Uptake of biogenic amines by two different mechanisms present in adrenergic granules *Brit. J Pharmacol* 1967 29 342—349
Lundborg, P and R. E. Stitzel, Effect of reserpine and protriptyline on the subcellular distribution of ^{3}H-metaraminol in the mouse heart. *Brit J Pharmacol.* 1967 b 30 379—384
Lundborg, P and B. Waldeck, Two different mechanisms for incorporation of ^{3}H metaraminol into the amine-storing granules. *J Pharm. Pharmacol* 1968 *18*, 762—764.
Shore, P A D Busfield and H. S Alpers, Binding and release of metaraminol Mechanism of norepinephrine depletion by α-methyl-M-tyrosine and related agents. *J Pharmacol exp Ther* 1964 *146* 194—199
Stitzel, R. E. and P Lundborg, Effect of reserpine monoamine oxidase inhibition on the uptake and subcellular distribution of ^{3}H-noradrenaline *Brit J Pharmacol.* 1967 93—104

Acta physiol. scand. 1968. 72. 396—403

From the Department of Physiology and the Department of Clinical Physiology
University of Göteborg, S eden

Extravascular Shunting of Oxygen in the Small Intestine of the Cat

By

MOGENS KAMPP OVE LUNDGREN and NILS JOHAN NILSSON

Received 12 July 1967

Abstract

KAMPP M., O LUNDGREN and N J NILSSON *Extravascular shunting of oxygen the small intestin of th cat* Acta physiol. scand. 1968. 72 396—403.

At resting" intestinal blood flow the enous appearance time of intra-arterially injected n cells labelled with methemoglobin exceeded by 1—2 sec that of oxygen administered in identical manner This obser tion is taken to indic te an extravascular shunting (counte current exchange of oxygen between the ascending and descending limbs of the mucou ascular loops located mainly in the villi. During vasodilatation induced by isopropylnor adrenaline or vasoconstriction induced by stimulation of the regional sympathetic fibres (impulsec no difference in enous ppearanc time between red cells and oxygen could l demonstrated The physiological significance of the findings of the present study is discussed

The existen e of a countercurrent exchange of materials between arterial and venou segments of capillary essels is firmly established in the mammalian kidney and ir th swimbladder of many fishes (for recent eviews see Lever 1965 and Fange 1966 respectively The countercurrent mechanism is dependent upon the close associ ation of the arterial and enous limbs of a ascular loop Such special ascula anatom seems tc be present in the intestinal illus of the cat and many othe mammals in which ascending non-branching central arterioles are surrounded b a descending dense subepithelial capillary network Heller 1872 Nasoka 19" Spanner 1932 Jacobsson and Noer 1952 The distance between the arterial and venous parts of thi asc lar loop estimated to be 10—20 μm and can be tra ersed by easily diffusible lipid soluble agents such as oxygen and krypton in less than one second (Kety 1951)

Experimental data which might be interpreted as evidence for countercurrent exchange in the intestine of the cat was recently presented by Kampp, Lundgren and Sjöstrand (196 In an attempt to determine blood flow distribution in the intestine by analysing th limination curve of intra-arterially injected krypton[85] they registered, at low otal intestinal blood flow a large and very fast initial component, a fact which uggested a shunting of krypton through the tissue. Theoreti-

such a shunt could be intravascular or extravascular. The present study was ...taken to differentiate between these two possibilities by comparing at various ...s of vascular smooth muscle tone the venous appearance time of oxygen with ... of red cells after injection into the superior mesenteric artery.

... preliminary ...eport of this study has recentl... been published (Kampp, Lund... and Nilsson 1967).

Methods

Operative procedure

... detailed description of the operative procedure the reader is referred to the paper by ...mpp, Lundgren and Sjöstrand (1967).

...bservations were carried out on 8 ... anesthetized ... hloralose (40—70 mg/kg) ... cats had been depr...ed of food for ... least 24 hou... and had no obvious signs of intestinal ...ection. The experiments wer... performed on intestinal segmen... weighing 30—50 g (mainly ...) prepared f...ee from adjacent intestinal ...sue. The mesenteric ...ein, ...hich in such a ...eparation drains all the blood from the in...es...nal segment and its lymph nodes, was ...ulated and connected to ... drop recorder ... ordinate ...ter. The blood ... returned to the animal ... the jugular ... Mea... ...erial blood pressure was recorded from ... left femoral artery by means of ... ...ometer. One of the mesenteric arteries supplying the intestine ... cannulated ... polyethylene catheter (PE 10) to permit ... intra-arterial injections ... ... could be ligated without interfering ... the blood suppl... ...

Intra-arterial infusions of isoproterenol ... ... intestinal vasodilatation, ... given ... a catheter (PE 10) inserted ... ... fashion into the superior mesenteric ... Atropine ... given ... mg/kg and ... nerves were cut bilaterally. The ... adrenal gland ... denervated and the ... ...ded from the circulation by ligatures ... those experiments in which ... sympathetic fibres was performed. In ... experiments ... ... constrictor fibres were stimulated ... bilaterally both adrenal glands ... excluded ... given hydrocortisone (Solu-... ... 5 mg/kg ...). The distal end ... splanchnic nerves were mounted on ... electrodes and ... stimulated ... ... pulses 5—7 ... 5 msec ... ... delivered ...

B. Oximetric ...

Through the thin ...ather... ... ...ppl... the intestine (see abov...) three ... of blood prepa... ... Blood ... equilibrated with pure oxygen (below ... ... volume of 1 per cent sodium nitrite ... followed by three ... methemoglobinemic cells (below ... methemog... ... The presence of methemoglobin ... the blood ... ... the extinction ... Microscopic ... ... no detectable alteration in size or shape ... ...ixed blood". Densitometric deflec... ... volume of methemoglobin ... ... described blood samples ...

The ... ...d from the intestine was recorded ... ... cuvette densitometer previously desc... ... 3.7 ml min through a thin pol... ... vein draining the intestinal ... isolated with interference ... ... of these wavelengths convert ... ...nction curves (Horecker 1943 ...) ... than ... reduced ... much greater at 625 than ... ... downwards a large at 62... ... the deflection at 625 ... ... deflection at 625 nm ... were inserted ...

the difference between them recorded. In the resulting tracing, therefore, oxygenation and methemoglobin addition gave deflections in opposite directions. Changes in venous hematocrit influenced only to a small extent the registration with this technique. The output from the photocells was registered on a Kipp Micrograph recorder. A test of the dynamic response of the apparatus as used in the present investigation gave a 90 per cent response time in 3 sec.

Results

A "Resting" blood flow. The results illustrated in Fig. 1 are representative for "resting" blood flow in the acutely denervated intestinal loop, *i.e.* venous outflow less than 60 ml/min × 100 g tissue. The upward deflection of the oximeter tracing in A indicates increased light transmission of the intestinal venous blood at 625 nm. This was due to increased oxygen saturation, induced by injecting 0.8 ml "oxygenated blood" over a period of 4 sec. The deflection began about 2.5 sec after the onset of the injection. The simultaneously recorded venous outflow amounted to 35 ml/min × 100 g.

Section B of Fig. 1 was registered about 6 min after the recording of A. When 0.2 ml of "methemoglobin blood" was diluted to 0.8 ml with isotonic saline and injected as before a downward deflection of the oximeter tracing was evident about 4 sec after the start of the injection. This indicated a reduced light transmission resulting from the presence of methemoglobinemic cells in the intestinal venous blood. The recorded venous outflow amounted to 42 ml/min × 100 g.

The difference of 1—2 sec in appearance time illustrated in A and B of Fig. 1 between "oxygenated blood" and "methemoglobin blood" was noted in most experiments when two equal injections were made at similar blood flow levels. To eliminate the difficulties inherent in comparing two different injections, the type of experiment shown in C of Fig. 1 was also performed (*cf.* Levy and Sauceda 1959): a well-mixed blood sample containing both "oxygenated blood" (1.2 ml) and "methemoglobin blood" (0.3 ml) was injected over a period of 6 sec. About 2.5 sec

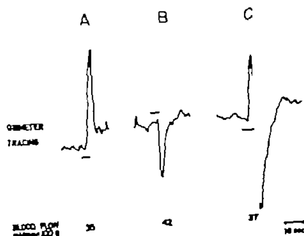


Fig. 1. Cat 2.7 kg. Tracings recorded from a cuvette oximeter connected to the venous outflow of an intestinal segment following intra-arterial injections of (A) "oxygenated blood", (B) "methemoglobin blood" and (C) mixture of both blood preparations. The horizontal bars mark the time of injection. Blood flow through the segment, measured concurrently with a drop recorder, is indicated below.

after the onset of the injection an upward deflection of the oximeter tracing was recorded, immediately followed by a downward deflection of somewhat greater magnitude. The venous outflow in this case was 37 ml/min × 100 g.

From the experiments described above, performed at "resting" blood flow levels, it is concluded that oxygen appears 1–2 sec earlier than methemoglobinemic cells in the mesenteric vein after an intra-arterial injection.

B. Vasodilatation. The effect of intestinal vasodilatation on the early appearance of oxygen in the venous effluent was studied in three experiments. Fig. 2 illustrates a representative experiment. The diphasic oximeter tracing of section A was registered at a resting blood flow level (29 ml/min × 100 g) after an intra-arterial injection of a blood sample containing both "oxygenated blood" (0.3 ml) and "methemoglobin blood" (0.1 ml). The light transmission at 625 nm was first increased (upward deflection) by the augmented oxygen saturation of the hemoglobin in the intestinal venous blood. As in C of Fig. 1 the upward deflection was interrupted by a downward deflection, caused by the reduction in light transmission from the presence of methemoglobinemic cells in the venous blood. A second upward deflection was also observed in this experiment. It reflects, in all probability, a higher oxygen saturation secondary to a transient increase of blood flow after the injection.

The oximeter registration of section B in Fig. 2 was obtained during a vasodilatation (70 ml/min × 100 g) induced by a constant, intra-arterial infusion of isopropylnoradrenaline. 0.4 ml of the "mixed blood" (same as in section A) now caused a single downward deflection of the oximeter. Thus, the initial upward deflection due to the early venous appearance of oxygen as described in Fig. 1 C and 2 A was absent at the increased blood flow level of Fig. 2 B. The oxygen saturation of the venous blood during the induced vasodilatation was, however, higher than at resting blood flow as judged by the colour of the venous blood and the position of the oximeter base line. The failure to demonstrate an increase in oxygen saturation upon intra-arterial injection of a mixture of "oxygenated blood" and "methemoglobin blood" might therefore possibly be ascribed to the

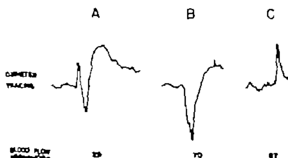


Fig. 2. Cat 3.6 kg. Tracings recorded from cuvette oximeter connected to the venous outflow of an intestinal segment following intra-arterial injections of "mixed blood" at resting blood flow (A) and during vasodilatation (B) induced by constant, intra-arterial infusion of isopropylnoradrenaline. In section C "oxygenated blood" was intra-arterially injected during vasodilatation. Blood flow through the segment, measured concurrently with drop recorder, is indicated below.

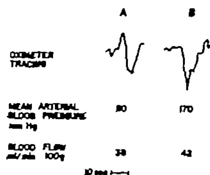


Fig 3 Cat 3 6 kg. Tracings recorded from a cuvette oximeter connected to the venous outflow of an intestinal segment following intra-arterial injections of "mixed blood" at resting blood flow (A) and during stimulation of the regional sympathetic vasoconstrictor fibres (5 V 5 msec, 4 imp/sec) (B) Mean arterial blood pressure and blood flow through the segment as indicated below

already high oxygen saturation of the venous blood. This possibility was excluded by the experiment in section C of Fig 2. Here 0.4 ml of "oxygenated blood" injected intra-arterially induced an upward deflection of the oximetric tracing at a venous outflow of 67 ml/min × 100 g Thus, the increased saturation of hemoglobin in the venous outflow in section B of Fig 2 was in all probability masked by a dominant downward deflection induced by methemoglobinemic cells, appearing simultaneously with the oxygenated cells.

It is concluded that the early appearance of oxygen in the intestinal venous blood as compared to labelled red cells which was observed during resting blood flow conditions in the intestine, was abolished by dilating the intestinal vascular bed.

Stimulation of the regional sympathetic vasoconstrictor fibres The effect of stimulating the regional sympathetic vasoconstrictor fibres on the early appearance of intra-arterially injected oxygen in the venous effluent of the intestine was studied in three experiments Fig 3 illustrates a typical experiment In section A, 0.9 ml of "mixed blood" (oxygenated blood and methemoglobin blood in volume proportions 2 1) was injected intra-arterially at a resting venous outflow of 38 ml/min × 100 g and a mean arterial blood pressure of 110 mm Hg This injection induced the characteristic diphasic oximeter deflection also shown previously in Fig 1 C and 2 A.

The same intra-arterial injection (1 0 ml of mixed blood) of section B in Fig 3 was performed during the steady state phase of regional vasoconstrictor fibre stimulation (4 imp/sec see Folkow *et al* 1964) when mean arterial blood pressure was 170 mm Hg and venous outflow 42 ml/min × 100 g It can be seen that a single downward deflection was recorded by the oximeter similar in appearance to that seen during induced intestinal vasodilatation (Fig. 2 B The oxygen saturation of the venous effluent from the intestine during stimulation was somewhat less than during prestimulatory control level as judged by the position of the oximeter base line. In accordance with the discussion in connection with Fig 2 B, it seems reasonable to ascribe the findings shown in Fig 3 B to the concomitant appearance of highly saturated hemoglobin and methemoglobinemic cells in the venous effluent.

It is concluded that the earlier appearance of oxygen in the intestinal venous blood as compared to labelled red cells, which was observed at resting blood flow was abolished during stimulation of the regional sympathetic vasoconstrictor fibres.

Discussion

It is well known that red cells pass through the vasculature of a tissue faster than plasma, because of the laminar flow of blood and the tendency of blood cells to move in the fast axial stream in the vessel. Experimental evidence in support of this assumption in the splanchnic area was presented by Chien (1963). Nevertheless, it could be shown in the present study that red cells labelled with methemoglobin appeared 1—2 sec later than oxygen in the venous effluent from the intestine at resting blood flow levels under conditions of simultaneous intra-arterial injection of a mixture of "oxygenated blood" and methemoglobinemic red cells.

This observation might be explained by a diffusion of oxygen along the long axis of the vessels, which is known to be facilitated by the presence of unsaturated hemoglobin (Scholander 1960). However, even if one takes this effect into account, it can be calculated that oxygen will appear only a fraction of second earlier than methemoglobinemic cells in the mesenteric vein after simultaneous intra arterial administration.

The results described in section A might be explained in terms of an arterio-venous plasma shunt based on extensive and highly efficient plasma skimming. However, no other data on the cat seems to support such a hypothesis since it would call for a degree of plasma skimming unknown to occur in any other tissue, including the kidney (Ulfendal 1962 a, b).

It might be argued that the methemoglobinemic red cells differed in their intravascular behaviour from normal red cells and passed through the intestinal vascular bed slower than red cells with hemoglobin. However, microscopic examination did not reveal any difference in shape between the two types of red cells. Furthermore, the results of Levy and Imperial (1961) indicate that methemoglobinemic and normal red cells pass equally fast through a vascular bed, to judge by the similar results obtained with both types of cells.

A more reasonable interpretation of the present data is the following: oxygen passing through the intestine leaves the intestinal vasculature at one point and then re-enters by diffusion at some other point downstream. This has been shown to occur in the renal medulla (Levy and Sauceda 1959, Levy and Imperial 1961, Aukland, Bower and Berliner 1964). From the present experiments it is not possible to draw any conclusions concerning the localization of such an extravascular short circuiting of oxygen. As indicated in the introduction, however, the special architecture of the vascular system of the intestinal mucosa and particularly of the villus, strongly suggests that countercurrent exchange may occur here. Furthermore, several observations have recently been reported using different techniques (Kampp and Lundgren 1966, Kampp, Lundgren and Sjostrand 196[illegible]) which all seem to support

the hypothesis of a countercurrent exchange, particularly of lipid soluble substances between the ascending and descending limbs of the mucosal vascular loops located mainly in the villi.

In a second type of experiment reported here it was demonstrated that the earlier appearance of oxygen in the mesenteric vein as compared to labelled red cells noted at "resting" blood flows was abolished by intestinal vasodilatation (Results, section B). There are two possible explanations for this finding. 1. It seems reasonable to assume that the linear flow velocity of blood increased during vasodilatation, and hence, the time available for countercurrent exchange decreased concomitantly. Thus a linear blood flow rate is reached at which no significant countercurrent exchange can take place (*cf.* Kampp and Lundgren 1967, Kampp, Lundgren and Sjöstrand 1967). 2. It was demonstrated by Kampp, Lundgren and Sjöstrand (1967) that during a vasodilatation induced by an infusion of isopropylnoradrenaline mucosal and submucosal vessels which seemed to be perfused at an extremely large blood flow rate, were opened up. It is possible that the appearance time of red cells, passing through such vessels, is as short as that of oxygen shunted extravascularly across the vascular loops of the mucosa.

In a third type of experiment it was demonstrated that the earlier appearance of oxygen in the mesenteric vein as compared to labelled red cells, which was regularly noted at "resting" intestinal blood flow levels, was abolished during stimulation of the regional sympathetic vasoconstrictor fibres (Results, section C). It is known from the work of Folkow *et al.* (1964 b) that vasoconstrictor fibre [illegible]ion reduces blood flow through the intestinal mucosa and probably diverts blood through submucosal vascular structures. The most plausible explanation for [illegible] observation described above therefore seems to be the opening up of vessels, the transit time of which was very short (compare the second alternative discussed above). It is, however, not possible to tell from the present experiment whether blood was diverted through the same vessels during sympathetic vasoconstrictor activation as during vasodilatation induced by isopropylnoradrenaline.

The possible physiological significance of the results of the present study will be shortly commented upon. The suggested countercurrent exchange of oxygen in the mucosa will create a gradient of decreasing oxygen tension in the villi from the bases towards the tips (countercurrent multiplication, *cf.* Aukland 1964). This may be a factor of importance in explaining the well known rapid turnover of the intestinal epithelial cells (see Wilson 1962, p. 12). In situations of increased oxygen need of these cells, i.e. during absorptive work, blood flow presumably increases and the countercurrent exchange mechanism becomes less efficient. Above a certain blood flow level, transit time in the hairpin vascular loops becomes too short to allow any significant short-circuiting of oxygen (see above). The metabolic processes in the intestinal cells are then freely supplied with oxygen. Furthermore, by means of the demonstrated extravascular oxygen shunt taking place at "resting" blood flows, oxygen which otherwise might have been "lost" into the intestinal lumen, is preserved for the liver, an organ demanding large amounts of oxygen.

This work was supported by grants from the Swedish Medical Research Council (B67 14X-16-03B) from Air Force School of Aerospace Medicine through the European Office of Aerospace Research (OAR) United States Air Force under Contract AF 61(052) 732, from U.S. Public Health Service (HE-05675-06) and from the Faculty of Medicine, University of Göteborg.

References

AUKLAND, K., Studies on intrarenal circulation with special reference to gas exchange. *J Oslo Cy Hosp.* 1964 *14* 115—146.

AUKLAND K., B. F BOWER and R. W BERLINER, Measurement of local blood flow with hydrogen gas. *Circulat. Res.* 1964. *14* 164—187

CHIEN, S., Cell volume, plasma volume and cell percentage in splanchnic circulation of splenectomized dogs. *Circulat. Res.* 1963 *12* 22—28.

FÄNGE, R., Physiology of the swimbladder. *Physiol. Rev* 1966. *46*. 299—322.

FOLKOW B D H. LEWIS, O LUNDGREN, S. MELLANDER and I. WALLENTIN, The effect of graded vasoconstrictor fibre stimulation on the intestinal resistance and capacitance vessels. *Acta physiol. scand.* 1964 a. *61* 445—457

FOLKOW B., D H. LEWIS, O LUNDGREN, S. MELLANDER and I. WALLENTIN, The effect of the sympathetic vasoconstrictor fibres on the distribution of capillary blood flow in the intestine. *Act physiol. scand* 1964 b. *61* 458—466.

HELLER, A., Über die Blutgefässe des Dünndarmes. *Ber sächs. Ges.* 1872 *24* 165—171

HORECKER, B. L., The absorption spectra of hemoglobin and its derivatives in the visible and near infra-red regions. *J biol. Chem.* 1943. *148*. 173—183.

JACOBSON, L. F and R. J NOER, The vascular pattern of the intestinal villi in various laboratory animals and man. *Anat. Rec.* 1952. *114* 85—101

KAMPP M and O LUNDGREN, Evidence for countercurrent exchange in intestinal villi. *Act physiol. scand* 1966. 68 Suppl. 277 103

KAMPP M. and O LUNDGREN, Blood flow and flow distribution in the small intestine of the cat as analysed by the Kr[85] wash-out technique. *Acta physiol scand* 1967 *00* 00—00

KAMPP M., O LUNDGREN and N. J NILSSON, Extravascular short-circuiting of oxygen indicating countercurrent exchange in the intestinal villi of the cat. *Experientia* (Basel) 1967 *23* 197—198.

KAMPP M., O. LUNDGREN and J. SJÖSTRAND On the components of the Kr[85] wash-out curves from the small intestine of the cat. *A physiol sc d* 1968 2 257—281

KETY S. S The theory and applications of the exchange of inert gas at the lungs and tissues *Pharmac Rev* 1951 3 1—41

LEMBERG, R. and J W LEGGE, *Hematin compounds and bile pigments* Interscience New York 1949

LEVER, A F The vasa recta and countercurrent multiplication *Acta med scand* 1965 *178* Suppl. 434 1—43

LE M N and E. INGRAHAM, Oxygen shunting in renal cortical and medullary capillaries *Amer J Physiol* 1961 200 159—162

LEVY M. N and O KASEDA, Diffusion of oxygen from arterial venous segments of renal capillaries. *Amer J Physiol.* 1959 *196* 1336—1337

NILSSON, N J A linearly responding dichroma den tometer for dye dilution curves *S nd J b Lab Invest* 1963 *15* Suppl 9 81 19

NIMURA, T 19 7 Cited by Spanner 1932

SCHOLANDER, P F Oxygen transport through hemoglobin solution *S nce* 1960 *131* 585—590

SP NER, R Neue Befunde über die Bl twege der Darmw nd und ihre funktionelle Bedeutung *Morph. Jb* 193 69 394—434

L UNDGREN, H R Distribution of red cell and plasma in rabbit and ca kidney *Act phy iol scand* 196 56 42—60

L UNDGREN, H R Hematocrit and hemoglobin concentr ion in venous blood drained from the outher ortex of cat kidney *Acta physiol sc d* 1962 b 54 61—69

WILSON T H *Intestinal absorption* W B Saunders Co Philad lphi London 19 2

Acta physiol. scand. 1968. 72. 404—411

From the Department of P thology the Gade Institute, School of Medicine
University of Bergen, Norway

Microcirculatory Changes and General Responses to 5-Hydroxytryptamine in Normothermic and Hypothermic Mice

By

KNUT SVANES

Received 12 July 1967

Abstract

SVANES, K. Microcirculatory changes and general responses to 5-Hydroxytryptamine in Normothermic and hypothermic mice. Acta physiol. scand. 1968. 72 404—411

5-hydroxytryptamine (5-HT) was injected into unanaesthetized, anesthetized normothermic and hypothermic (21—22 C) mice and the general effects of the injections were observed. In addition, conjunctival microscopy and hematocrit determinations were performed 5-HT was found t cause syndrome characterized by exophtalmos, respiratory distress, cyanosis, edema evacuation of bowels, sall ation, lethargy constriction and slow blood flow in the arterioles, dilatation and stasis i the enules, and hemoconcentration. I gene l the re to 5-HT was relatn ly mild in unanesthetized mice moderate in anesthetized normothermic mice and severe in hypothermic mice. The marked reaction to 5-HT und hypo in might be due to retarded elimination of 5-HT from the blood

In a previous investigation (Svanes 1966 b) low molecular weight dext gi en i.v. was found to cause an anaphylactoid reaction in hypothermic but not in normothermic mice. Evidence wa provided that the reaction was mediated thr gh the release of 5-hydroxytryptamine (5-HT) It was assumed that retarded el nmation of 5-HT at low temperat re might explain the increased reaction to dextran under hypothermia.

The present investigation was undertaken in order to find out if the relationship between drug effect and body temperature was the same for 5-HT as that which had been found for dextran 5-HT was given i.v into normothermic and hypothermic mice. The general effects of the injections were observed capillary microscopy was performed and hematocrit alues were determined.

Material and Methods

Animals. Male albino mic 3—8 months old were used

5-hydroxytryptamine (5-HT) (Serotonine creatinine sulphate, Koch-Light Laboratories Ltd) was used as 0.0015—1.5 % solutions w/v in isotonic saline and as injected during 1 min in doses of 0.15—150 mg/kg b.w (10 ml/kg b.w

Hematocrit The hematocrit values were determined in blood from the right ventricle of the heart, as described previously (Svanes 1966 b)

Capillary microscopy The microcirculation was studied in the capillary bed of the conjunctiva of the lower eyelid, as described previously (Svanes 1966 a)

Anesthesia, normothermia The mice were anesthetized with pentobarbital and kept at approximately normal body temperature (Svanes 1967) When 5-HT was injected, some groups of mice were under light anesthesia (respiratory frequencies between 120 and 160 per min, active corneal reflex), and some were under rather deep anesthesia (respiratory frequencies —100 per min and corneal reflex abolished)

Hypothermia The technique of hypothermia has been described previously (Svanes 196) The mice were anesthetized with pentobarbital and cooled to rectal temperature of 1 22 C. When 5-HT was injected, some groups of mice were under light anesthesia (respiratory frequencies 30—45 per min, corneal reflex present) and some were under rather deep anesthesia (respiratory frequencies 20—30 per min, corneal reflex abolished)

Results

Lethality general effects and changes in the microcirculation upon 5-HT injection

In this set of experiments 128 mice which were 5—8 months old and weighed 35 g were used. They were divided in 25 groups, in which the animals were pretreated and injected i.v. with 5-HT as indicated in Table I. Corresponding groups of unanesthetized, anesthetized normothermic and hypothermic mice were given 5-HT i.v. in doses of 150 mg/kg, 15 mg/kg, 1.5 mg/kg, 0.15 mg/kg and 0 mg/kg (saline). One 5-HT-treated and one saline-treated mouse were always examined simultaneously. The injection of 5-HT was given after 1 hr of anesthesia to the anesthetized normothermic mice, and after 1 2 hr of hypothermia to the hypothermic mice. The mice were killed 1 hr after the injection of 5-HT

Lethality As shown in Table I none of the unanesthetized and only one of the anesthetized normothermic mice died after the injection of 5-HT. Of the hypothermic mice all died after injection of 150 mg/kg. After injection of 5-HT in doses of 15, 1.5 and 0.15 mg/kg one mouse in each of the groups with hypothermic deeply anesthetized animals died. All deaths occurred within the first 5 min after the injection.

General signs The signs observed in mice after injection of 5-HT are [illegible] in Table II. In unanesthetized mice 150 mg/kg 5-HT caused [illegible] subsided during the last half hour of the experiment [illegible] persisted throughout the experiment. 5-HT in doses of 15, 1.5 and 0.15 mg/kg caused minimal or no responses — In anesthetized normothermic [illegible] 5-HT caused severe changes. Capillary microscopy was difficult [illegible] carry out because of extreme exophthalmos. Pharyngeal aspiration [illegible] out repeatedly because of salivation. However the mice [illegible] half hour of the experiment. 5-HT in the dose of 15 [illegible] response and 1.5 and 0.15 mg/kg 5-HT caused no [illegible] mice died after the injection of 150 mg/kg 5-HT [illegible] jection of 5-HT or after —3 min with irregular [illegible]

TABLE I Lethality after I. Injection of 5-hydroxytryptamine in mice.

Additional treatment	5-hydroxytryptamine (mg/kg)				
	150	15	1.5	0.15	0 (saline)
None	0/4	0/4	0/4	0/4	0/16
Normothermia, light anesthesia	0/3	0/3	0/3	0/3	0/12
Normothermia, deep anesthesia	1/3	0/3	0/3	0/3	0/12
Hypothermia, light anesthesia	3/3	0/3	0/3	0/3	0/12
Hypothermia, deep anesthesia	3/3	1/3	1/3	1/3	0/12

Deaths/number injected.

TABLE II General signs follo ing injection of 5-hydroxytryptamine in mice.

Additional treatment	5-HT	Exophtalmus	Respiratory distress	Cyanosis	Erythema of the skin	Edema of the lips	Evacuation of bowels	Salivation	Lethargy
	150	+	0	0	+	+	++	0	++
	13	(+)	0	0	+	+	+	0	
	1.5	0	0	0	+	0	0	0	+
	0.15	0	0	0	0	0	0	0	0
Anesthesia, normothermia	150	+++	++	++	0	+	++	++	
	15	++	+	+	0	+	+	(+)	
	1.5	(+)	(+)	0	0	0	0	0	
	0.13	0	0	0	0	0	0	0	
Anesthesia, hypothermia	150	(All mice died after injection of 150 mg/kg 5-hydroxytryptamine)							
	13	+	+	++	0	+	0	0	
	1.5	(+)	(+)	+	0	0	0	0	
	0.15	(+)	0	0	(+)	0	0	0	

served on the thoracic wall some min after respiration had ceased. 5-HT in the dose of 15 mg/kg caused severe cyanosis, and the mice were in poor condition t the end of experiment. 5-HT in the dose of 1.5 mg/kg caused a relatively mild response, and 0.15 mg/kg *caused none.*

Capillary microscopy. In anesthetized normothermic mice the injection of 150 mg/kg 5-HT caused an extreme exophthalmus which prevented capillary microscopy. After injection of 15 mg/kg 5-HT the following observations were made. Just after the injection no blood circulation was observed at all, or slow flow might be seen in a single metarteriole. The venules were markedly dilated and contained stagnant concentrated blood (stasis) (Zweifach 1965 p. 180). Most of the true capillaries were also dilated and filled with stagnant blood. After 20—30 min blood circulation was restored in most of the capillary vessels. However, usually some venules and true capillaries with stagnant blood were observed throughout the experiment (1 hr). A single petechial hemorrhage might be seen.

5-HT in doses of 1.5 mg/kg and 0.15 mg/kg did not cause venular stasis in anesthetized normothermic mice.

Hypothermic mice died short after the injection of 150 mg/kg 5-HT. Capillary microscopy immediately after the injection disclosed total circulatory standstill. A few true capillaries and venules contained stagnant blood. Stasis was not observed.

After injection of 15 mg/kg and 1.5 mg/kg of 5-HT into hypothermic mice, the following observations were made: Just after the injection slow granular flow was observed in one or several metarterioles which were constricted. The venules were not dilated and contained some stagnant blood. During the next 5 min the blood flow continued in the metarterioles, while no flow was observed in the venules. The venules became increasingly distended and packed with erythrocytes, until they were maximally dilated and filled with a homogenous mass of stagnant erythrocytes (stasis). Simultaneously an increasing number of true capillaries became filled with stagnant erythrocytes. After injection of 15 mg/kg 5-HT the blood flow was not restored at all in some mice during the first hr after the injection. In others slow blood flow was observed in a few vessels after 1 hr. After injection of 1.5 mg/kg 5-HT the blood flow was restored partly or completely 15—30 min after the injection (Table III).

Usually the injection of 0.15 mg/kg 5-HT into hypothermic mice did not cause venular stasis. However, in one mouse 0.15 mg/kg 5-HT caused the following changes. Immediately after the injection the conjunctiva was ischemic. Slow granular blood flow was observed in 3 metarterioles while no flow was seen in the

TABLE III. Duration (in minutes) of venular stasis after injection of 5-hydroxytryptamine in mice

Additional treatment	5-hydroxytryptamine mg/kg			
	15	1.5	0.15	0
Anesthesia, normothermia	20—30	0	0	0
Anesthesia, hypothermia	60	15—30	0—6	

TABLE IV. Hematocrit values 1 hr after i. injection of 5-hydroxytryptamine in mice.

Additional treatment	5-HT	Number of mice	Hematocrit values (mean ± S.D.)
None	150	4	57.5 ± 2.4
	15	4	51.5 ± 2.4
	1.5	4	50.0 ± 0.8
	0.15	4	48.5 ± 4.2
	0	16	48.2 ± 2.4
Anesthesia, normothermia	150	5	53.2 ± 2.7
	15	6	53.8 ± 2.5
	1.5	6	47.7 ± 4.4
	0.15	6	45.8 ± 3.3
	0	24	45.2 ± 1.8
Anesthesia, hypothermia	150	0	
	15	5	72.6 ± 6.7
	1.5	5	65.0 ± 4.5
	0.15	5	52.4 ± 1.5
	0	24	48.7 ± 3.3

venules. During 6 min an increasing erythrostasis occurred in the venules and true capillaries. Then the blood flow was suddenly restored in the distal venules, followed by circulation in the collecting venules and true capillaries.

Changes in hematocrit values upon 5-HT injections

In the set of experiments described above the hematocrit values were determined 1 hr after the injection of 5-HT. The values were not found to be much influenced by the depth of anesthesia. Therefore values from mice under deep and light anesthesia were considered together. The results are given in Table IV. In both unanesthetized, anesthetized normothermic and hypothermic mice did injections of 5-HT cause increases in hematocrit. A statistically significant correlation between doses of 5-HT and hematocrit values after 1 hr was found for unanesthetized ($r = +0.80$, $P < 0.001$), anesthetized normothermic ($r = +0.43$, $P < 0.05$) and hypothermic mice ($r = +0.91$, $P < 0.001$).

A second set of experiments was carried out to determine the hematocrit values at different intervals after the injection of 5-HT. 5-HT was given i. in doses of 1.5 mg/kg and 15 mg/kg since in these doses 5-HT caused vascular stasis without killing the mice. Eighty-one mice which were 3—4 months old and weighed 28—32 g, were used. They were pretreated and injected as indicated in Table V. Only light anesthesia was used. The injection of 5-HT was given after 1 hr of anesthesia to the anesthetized normothermic mice, and after 1/2 hr of hypothermia to the hypothermic mice. Groups of mice were killed 10, 20 and 30 min respectively after the injection of 5-HT.

TABLE \ Hematocrit alues (mean of 3 observations with range) 10, 20 and 30 min after injection of 5-hydroxytryptamine in mice.

Additional treatment	5-HT (mg/kg)	Minutes after injection of 5-hydroxytryptamine		
		10	20	30
	15	60 (57—63)	60 (56—62)	56 54—58
	1.5	55 (52—56)	52 (49—56)	53 51—57
	0	50 (48—53)	51 (45—54)	50 46—53
Anesthesia, normothermia	15	56 (5.—58)	56 (5.—58)	57 (53—60
	1.5	49 (46—53)	50 (48—51)	47 (43—52
	0	43 (42—44)	44 (41—46)	44 (41—47
Anesthesia, hypothermia	15	61 (59—63)	60 (59—62)	70 (67—72)
	1.5	63 (6C—55)	61 (56—67)	61 (58—64
	0	49 (48—50)	47 (46—49)	49 (43—53)

The results are shown in Table \ Two-way analyses of ariance were used in the statistical treatment of the results recorded in the table. In unanesthetized mice 1.5 mg kg 5-HT caused a slight (not significant) and 15 mg kg 5-HT a marked increase in hematocrit ($P<0.005$) In anesthetized normothermic mice 1.5 mg kg 5-HT caused a moderate increase ($P<0.01$) and 15 mg kg 5-HT caused a marked increase in hematocrit ($P<0.005$) In hypothermic mice both 1.5 mg kg and 15 mg kg 5-HT caused marked increases in hematocrit ($P<0.005$)

Fig. 1 illustrates the alterations in hematocrit alues with time during the first hr after the injection of 5-HT in doses of 1.5 mg kg and 15 mg kg b w The 10 20

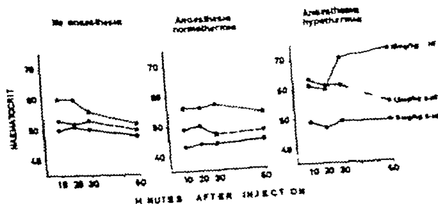


Fig. 1 Hematocrit alues after injection of 5-hydroxytryptamine in mice
5-HT 15 mg kg
— — — — 5-HT 1.5 mg kg
———— Saline control
The points represent mean alues, 10 20 and 30 min alues taken from T bl \ 60 min taken from T ble IV

and 30 min values are taken from Table V the 60 min values from Table IV In general 5-HT caused greater and more prolonged increase in hematocrit in anesthetized than in unanesthetized mice, and greater and more prolonged increase in hypothermic than anesthetized normothermic mice.

Discussion

In the present investigation the i.v injection of 5-HT into mice was found to cause a syndrome characterized by exophthalmus, respiratory distress, cyanosis, edema of the lips, evacuation of bowels, salivation, lethargy venular stasis and hemoconcentration. In general the reaction to 5-HT was relatively mild in unanesthetized mice, moderate in anesthetized normothermic mice and severe in hypothermic mice.

5-HT given i.v into mice caused constriction and slow blood flow in the metarterioles and dilatation and stasis in the venules of the conjunctiva. Veins and arteries are not visible in the conjunctiva of the lower eyelid of mice.—Rowley (1964) found that 5-HT injected into rat skin caused local constriction of veins and dilatation, increased permeability congestion, and decreased blood flow in venules" Sometimes 5-HT was also found to cause arterial constriction.—The result of Rowley appears to be in accordance with that of the present investigation in mice

The dilatation of the venules indicates that the hydrostatic pressure in the venules might be elevated (Law of Laplace) This is in accordance with the observation of Haddy *et al* (1957) that 5-HT given intra arterially into dogs elevated small vein pressure and consequently also the venular pressure.—5-HT has also been found to increase the permeability of the venules (Majno *et al.* 1961) Increased intravascular pressure as well as increased permeability of the venular wall might thus have contributed to the venular stasis after 5-HT injections in the present study

In the present experiments the microcirculation was studied only in the conjunctiva. However the general hemoconcentration caused by 5-HT indicates that the vascular changes observed in the conjunctiva after injection of 5-HT may be representative for many capillary beds

5-HT caused more severe symptoms in anesthetized normothermic than in unanesthetized mice The reason for this is not known. However some evidence has been provided that pentobarbital anesthesia might cause a condition of stress in normothermic mice (Svanes 1964) Under stress the adrenals liberate increased amounts of catecholamines (Euler 1955/56) and catecholamines have been found to increase the effect of 5-HT on the microcirculation (Zweifach, 1964)

I.v injected 5-HT is normally eliminated rapidly from the circulating blood by enzymatic deamination and binding in various tissues (Axelrod and Inscoe 1963, Davis and Wang 1965) The enzymatic deamination of 5-HT can be expected to be considerably retarded under hypothermia.—The distribution of a drug may also be retarded under hypothermia as a consequence of reduced cardiac output (Brendel, Albers and Usinger 1958, Bullard 1959) and retarded diffusion of drugs through

cell membranes and body fluids (Giese 1962, p. 202). Probably the binding of 5-HT in the tissues is therefore also retarded under hypothermia. The increased reaction to 5-HT in hypothermic mice can therefore probably be explained as a result of retarded elimination of the agent from the blood.

The reaction of mice to i.v. administered 5-HT has much in common with their reaction to low molecular weight dextran (Svanes 1966 b). The relationship between induced effect and body temperature was also the same for the two substances. The present experiment provides support, therefore, to the hypothesis that the marked reaction to low molecular weight dextran in hypothermic mice may be due, at least in part, to the release of 5-HT.

The present investigation was supported by grants from L. Meltzers' Høyskolefond and Dr. Nils Henrichsens og hustru Anna Henrichsens legat.

References

Axelsson, J. and J. K. Inscoe, The uptake and binding of circulating serotonin and the effect of drugs. *J. Pharmacol. exp. Ther.* 1963, *141* 161–165.

Barthel, W., C. Albers, and W. Usinger, Der Kreislauf in Hypothermie (Die einzelnen Kreislaufgrössen und ihre Koordination während der Auskühlung). *Pflügers Arch. ges. Physiol.* 1958, 266 341–356.

Bullard, R. W. Cardiac output of the hypothermic rat. *Amer. J. Physiol.* 1959 *196* 415–419.

Davis, R. B. and Y. Wang, Rapid pulmonary removal of 5-hydroxytryptamine in the intact dog. *Proc. Soc. exp. Biol. (N.Y.)* 1965, *118*, 797–800.

Euler, U. S., Stress and catechol hormones. Pp. 125–137 in Selye, H. and Heuser, G. Fifth annual report on stress 1955–56. New York, Md publications, Inc. 815 pp.

Giese, A. C. Cell Physiology 2nd Ed. Saunders, London, 1962.

Haddy, F. J., M. Fleishman, and D. A. Emanuel, Effect of epinephrine, norepinephrine and serotonin upon systemic small and large vessel resistance. *Circulat. Res.* 1957 *5* 247–251.

Majno, G., G. E. Palade, and G. I. Schoefl, Studies on inflammation II. The site of action of histamine and serotonin along the vascular tree. A topographic study. *J. biophys. biochem. Cytol.* 1961 *11* 607–626.

Rowley, D. A. Venous constriction as the cause of increased vascular permeability produced by 5-hydroxytryptamine, histamine, bradykinin and 48/80 in the rat. *Brit. J. exp. Path.* 1964, *45*, 56–67.

Svanes, K., Studies in Hypothermia. III. The influence of prolonged deep hypothermia and of barbiturate anesthesia on the blood leukocytes in mice. *Acta anaesth. scand.* 1964 *8* 167–179.

Svanes, K., Studies in hypothermia. VI. Intravascular aggregation in hypothermic mice. *Acta anaesth. scand.* 1966 a. *10* 123–131.

Svanes, K. Studies in hypothermia. VII. Anaphylactoid reaction to low-molecular-weight dextran in hypothermic mice. *Acta anaesth. scand.* 1966 b. *10* 133–146.

Svanes, K., Studies in hypothermia. VIII. Relation between depth of pentobarbital anesthesia, capillary circulation and formation of turpentine edema, under hypothermia and normothermia. *Acta anaesth. scand.* 1967 *11* 1–13.

Zweifach, B. W. Microcirculatory aspects of tissue injury. *Ann. N.Y. Acad. Sci.* 1964 *116* 831–838.

Zweifach, B. W. Microvascular aspects of tissue injury. Pp. 161–196, in Zweifach, B. W., Grant, L., and McCluskey, R. T. The inflammatory process. New York, Academic Press, 1965 931 pp.

Acta physiol scand 1968, 72, 412—424

From the Institute of Anatomy and Histology, Department of Pharmacology and Department of Obstetrics and Gynecology at Malmö, University of Lund, Sweden

Changes in the Amount of Adrenergic Transmitter in the Female Genital Tract of Rabbit During Pregnancy

By

E. Rosengren and N.-O. Sjöberg

Received 21 July 1967

Abstract

Rosengren, E. and N.-O. Sjöberg. *Changes in the amount of adrenergic transmitter in the female genital tract of rabbit during pregnancy.* Acta physiol. scand. 1968. 72, 412—424.

The noradrenaline present in the female reproductive organs — ovary, oviduct, uterus and vagina — of the rabbit is stored in adrenergic nerves. Fluorescence microscopy revealed that these nerves are related to the vascular bed and the smooth muscle walls; besides the muscular nerves the ovary also contains adrenergic nerves unrelated to the muscle. The adrenergic innervation of the genital organs originates from sympathetic ganglia located near the pelvic organs, as well as from pre- and paravertebral ganglia. During the former half of pregnancy a marked increase in neuronal noradrenaline occurs in the reproductive organs, except in the ovary. In the uterus and the vagina this increase is followed by a dramatic decrease in total noradrenaline during the latter half of pregnancy; by the end of pregnancy the uterus is almost completely devoid of noradrenaline-containing sympathetic nerves. The findings are discussed in terms of an humoral influence on the adrenergic neurons supplying the female reproductive tract.

During the last few years much interest has been devoted to the alterations occurring in uterine catecholamine levels under the influence of female sex hormones as well as during pregnancy. In the non-pregnant rabbit Cha *et al.* (1965) reported the presence of significant amounts of both noradrenaline and adrenaline, the concentrations of which were found to decrease significantly during early pregnancy. But recent data make it questionable whether adrenaline even occurs at all in the uterus of non-pregnant rabbits (Miller and Marshall 1965, Owman *et al.* 1966). The fall in uterine noradrenaline concentration (per unit of weight) during pregnancy can well be explained by the pronounced increase in weight of the organ during this state. Moreover Miller and Marshall (1965) found no significant alteration in the total amount of noradrenaline in the uterus following administration of estrogen alone or combined with progesterone.

Recent detailed fluorescence histochemical investigations (Owman and Sjöberg 1966, Owman *et al.* 1966) have established that noradrenaline in the female genital tract of the rabbit is stored in sympathetic nerves related to blood vessels and the smooth muscle coats. Also adrenergic nerves unrelated to blood vessels were found in the ovary The muscular adrenergic nerves of the uterus, vagina and partly also of the oviduct constitute a special kind of short sympathetic neurons arising in pe pheral ganglia at the utero-vaginal junction. The remaining muscular adrenergic nerves of the oviduct as well as the vascular adrenergic innervation (Sjöberg 1967) in the above-mentioned organs and in the ovary originate in pre and paravertebral sympathetic ganglia.

Against the background of the detailed knowledge of the adrenergic innervation pattern in combination with the use of histochemical and chemical methods for analysis of the adrenergic transmitter new possibilities are available for studying the female genital tract for changes in the noradrenaline content under hormonal influence, including pregnancy This report presents strong evidence for a pronounced alteration of the noradrenaline level in the rabbit female reproductive organs during various stages of pregnancy

Material and methods

The material consisted of adult nulliparous albino rabbits, [illegible] eighing between 2 0—3 2 kg, and kept separated from males after the weaning period. All the animals were obtained from the same breeder and fed on the same diet of standard pellets (S.A.N-bolagen, Sweden [illegible] turnips and ater ad lib

Some of the animals wer mated the first da after observed copulation as designated day 1 of pregnancy G oups of animals [illegible] er killed at three stages of pregnancy (in this strain of animals parturition occurred ithin 31 days)

Group A 4—1 days of pregnancy (7 animals

Group B 15—24 day of pregnancy (14 animals)

Group C 25—30 day of pregnancy (11 animals

The results wer compared ith those obtained in 21 non-pregnant animals The experi ments ere performed during December to May All animals ere killed by [illegible] injection of air

For histochemical demonstration of the adrenergic transmitter according to Falck and Hillarp (Falck 1962 Falck et al. 1962, Corrodi and Hillarp 1963 1964 the en re genital trac [illegible] removed en bloc immediately after the animals had been killed I some of the animals numerous large representativ pieces were taken f om [illegible] ious parts of he ovary oviduct, uterus and agina. The est of the animals er [illegible] ed also for bioche [illegible] nal ses see below I order not to interfere ith the total amine [illegible] on en of the orga [illegible] only few and minute pieces ere excised f om the animals used fo bi [illegible] al [illegible] al [illegible] All speci me [illegible] er quenched to the temperatur of liquid nitrogen and subseque [illegible] ted [illegible] ordi [illegible] he proc dur described in detail by Falck and Owman 1965 Under the opt al [illegible] d [illegible] ed [illegible] holamines emit an intense green light [illegible] he [illegible] he fluorescence of 5 1 d ox [illegible] ptamine is [illegible] llow

Dopamine noradrenaline and adrenaline in the ovaries oviduc [illegible] ter [illegible] nd [illegible] agina [illegible] measured fluorimetrically according to he method of Bertle et al 1958 as modified by Häggendal (1963) Determinations of these substanc es [illegible] ach animal er made on homogenates of the agina, both ovaries both oviduct [illegible] nd [illegible] h [illegible] er

Results

Non-pregna t [illegible] *mal* T [illegible] tment of the aric s reproductiv organ w th formal dehyde induced a pecific fluorescence in nerves which emitted a green l [illegible] character tic of [illegible] techolamines (see F lck nd Owm n 1965 Ther wa [illegible]

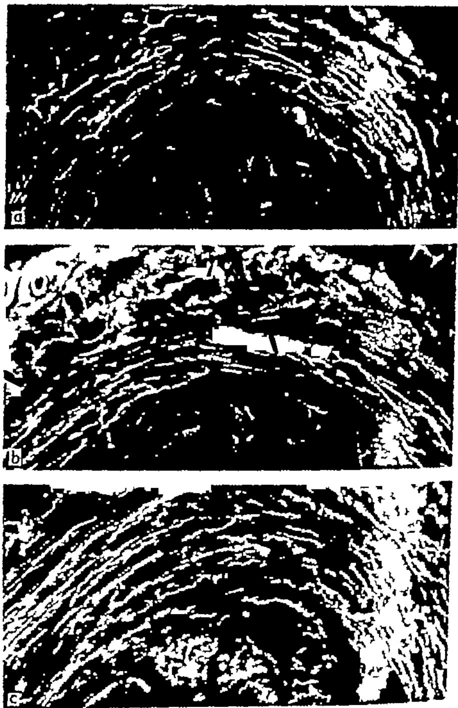
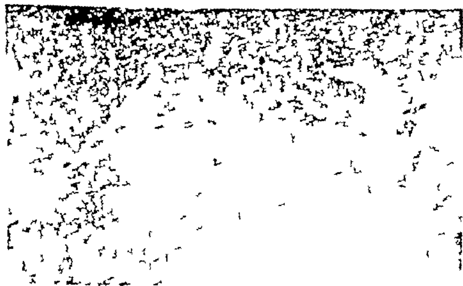


Fig. 2 Isthmic parts of the oviduc ... ns ma section 160
a Non-pregnant ... imal Moderat ... umber of fl ... e en ... erv ... terminals in the circula muscle coat
b) 10 days of pregnanc ... Group A ... 4 d ... nt ... se ... he densit ... of adrenergic muscular nerves
(c 14 days of pregnanc ... Group B Fluo esc ... terminal with ... distribution and densit ... similar to that in a







(c) 27 days of pregnancy cervical region Group C. The cervical region contains distinctly larger number of fluorescent nerves than the uterine horns. The morphology of the nerves is similar to that in Fig 3b. 160 ×

nsity of the adrenergic innervation was larger in the cervical region than in the n of the uterus, a finding also made in group A.

In this stage of pregnancy the fluorescence intensity of the uterine nerves did not differ from that in non-pregnant animals. The total noradrenaline content (Fig 1c) had decreased by one third (P<0.001) compared with the early pregnancy group, although it was still significantly higher than in non-pregnant animals (0.001< P<0.01

The *vagina* too showed a further increase in weight (Fig. 1d) while the total noradrenaline (Fig 1d) did not differ from that in group A (P>0.05) but was higher than in non-pregnant animals (P<0.001) The density of the adrenergic innervation by way of fibres with a normal fluorescence intensity appeared even more reduced (Fig 4c than in the preceding group of animals

Group C 25—30 days of pregnancy Also during late pregnancy the ovarian weight remained as high as before (Fig 1a and the noradrenaline content persisted at the same low level as in the non pregnant animals (Fig 1a) The number and distribution of adrenergic nerves were the same as in the two earlier pregnancy groups.

The results of the histochemical and fluorimetric Fig 1b) analyses of the *oviduct* were largely the same as in groups A and B Neither had the organ increased significantly in weight.





( ) 17 days of pregnancy Group B. The density of adrenergic innervation in the gina more reduced than i the preceding group of animals owing t further increase weight

uterine horns failed to demonstrate any nerves in the smooth muscle layer or the vascular bed. Only occasionally were one or two varicose fibres recognized (Fig 3b) it was difficult to decide wh ther they were related to the muscles or to blood vessels. Characteristically the fluorescence in such fibres was weaker than in non pregnant animals and occurred nly in the varicosities, the intervaricose segments of the fibre being essentially non-fluorescent

The cervical region contained a distinctly larger number of fluorescent nerves (Fig 3c) than that found in the uterine horns. They were morphologically similar to those mentioned above

In agreement with the finding of a conspicuously small number of nerves with a reduced fluorescence in the terus, only a small amount of noradrenaline (Fig 1c) could be demonstrated fluorimetrically (in comparison with non-pregnant animals $0.001 < P < 0.01$)

The total noradrenaline content of the *agina* tended to decrease further (Fig 1d) during the last phase of pregnancy ($0.0 < P < 0.05$) although it was still higher ($0.01 < P < 0.02$) than in non-pregnant animals. As seen in the fluorescence microscope the adrenergic nerve fibres wer even parser than in group B. This apparently reflects a true decrease in the densit of adrenergic innervation since the weight of the organ was about twice that in group B. The morphology and general arrangement of the fluorescent nerves was essentially unchanged, although the intensity was sometimes reduced.

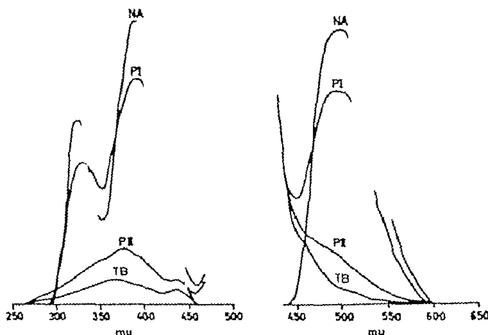


Fig. 5 Excitation (left) and emission (right) spectra of thentic norad enaline (NA) extract from terus of animals in Group B (P I) and G oup C (P II) Tissue blank (TB)

Mod l periments In the fluorimetric analyses of uterine tissue the spectral characteristics of the organ extract from group C differed from those of the other groups, including non-pregnant animals, as well as from that of authentic noradrenaline (Fig 5) This discrepancy may be due to the amounts of noradrenaline in aliquots used for final analysis being too small to produce distinct maxima in the excitation and emission spectra. When a uterine horn from a non-pregnant animal vas homogenized together with that from a group C animal (2 expts.) the fluorimetric reading of the aliquots from the extract evealed normal spectral characteristics and thereby supported the above mentioned assumption The same esult was obtained when 1 μg noradrenaline was added to a 10 ml aliquot of the extract f om a group C uterine horn (2 expts.) But these experiments do not exclude the possibilit f the difference n spectral characteristic being due to some interfering material present n the uterus only during lat pregnancy

Discussion

In the combined hemical and histochemical tudies of the adrenergic transmitter level in the reproductiv tract of the rabbit in relation to hanges in weight of the organ during pregnancy the following observ tions of interest were made

In spite of considerable increase in the ovarian weight during the first stag

pregnancy after which the weight remains at a high level the total amount of noradrenaline is unchanged, compared with that in non-pregnant animals. The appearance of the adrenergic innervation seemed essentially unchanged during pregnancy which was expected since the weight increase is due to isolated changes in the organ, i.e. development of corpora lutea.

Also the oviduct increased markedly in weight during pregnancy The increase was accompanied by a rise in the total level of noradrenaline. Fluorescence microscopy did not reveal any particular alteration in the arrangement or transmitter content of the sympathetic innervation. Hence, the results demonstrated that in pregnant rabbits the innervation of the oviduct is at least as dense as in non-pregnant animals. This innervation pattern remains unaltered throughout gestation.

During the first two weeks of pregnancy the noradrenaline concentration *per unit of weight* of the uterus was significantly lower than in non pregnant animals. In the fluorescence microscope, this manifested itself as a reduction in the density of adrenergic innervation. However a marked increase in the *total* noradrenaline content was nevertheless observed. From the third week of pregnancy a dramatic change in the transmitter content was noted. There was a steady increase in the weight of the uterus. However the total noradrenaline content markedly decreased to reach levels below those found in non-pregnant animals. In fact, during the last period of pregnancy only few if any fluorescent nerves could be visualized in any part of uterus except in the cervix, where the number of the adrenergic nerve terminals and their transmitter content were lower than in non pregnant animals as ll as in the major part of pregnancy

A similar pattern of concomitant changes in the organ weight, noradrenaline content and density of adrenergic innervation was also noted for the vagina, although the changes were less conspicuous than in the uterus. Thus, a certain number of adrenergic nerves with a reduced fluorescence intensity still persisted in the vagina, even during late pregnancy

The present experiments demonstrated that during the first stage of pregnancy there is a considerable increase in the noradrenaline content of the oviduct, uterus, and vagina. The result from previous investigations on the sympathetic innervation of the female reproductive tract (Owman and Sjoberg 1966, Owman *et al.* 1966) together with the close correlation between the histochemical and chemical data in the present study indicate that the changes observed pertains to a neuronal store of noradrenaline. During the pregnancy it was difficult clearly to establish whether the *intensity* in the fluorescent nerves already present in non-pregnant animals had increased. However it could be demonstrated that the *number* of nerves with a detectable fluorescence had increased. This would mean that in non-pregnant animals adrenergic nerves are present but with a transmitter content too low for histochemical demonstration (unless the increased number of fibres during pregnancy represent newly formed axon branchings

This assumption is in agreement with the increase in the density of adrenergic nerves, the increase in their transmitter content and the increase in total noradrena-

line occurring particularly in the uterus and vagina after treatment of rabbits with estrogen (Sjöberg 1967). Moreover, a variation of transmitter content, probably owing to changes in functional conditions has been demonstrated in sympathetic nerves also at other sites (Owman 1967, Alm *et al.* 1967). There is thus strong evidence that neuronal noradrenaline in the female genital tract increases as a consequence of the specific hormonal status occurring in association with pregnancy.

During the latter half of gestation there was a marked decrease in the noradrenaline content in the vagina and, particularly in the uterus, where hardly any adrenergic nerves could be detected histochemically during the last days of pregnancy. Fluorescence microscopy indicates that the reduction is a combined effect of a decreased transmitter content of the individual axons and a reduced number of fluorescent adrenergic nerves. These two changes in the adrenergic innervation may be explained in different ways. (a) A continuous diffuse enlargement of the vagina and the uterine horns during pregnancy may interfere mechanically with the axons to cause a reduction in their number. This would help to explain why the reduction in the number of fluorescent nerves is less conspicuous in the oviducts and in the uterine cervices, whose increase in size is less prominent during pregnancy. (b) A continuous influence by hormones during pregnancy might change the synthesis, storage or release mechanisms in the neurons resulting in a net reduction in the transmitter content. Rudzik and Miller (1962) demonstrated that estrogen initially produces an increase in uterine catecholamines in rats followed by a decrease on further treatment. (c) The balance in the transmitter content of the nerves might also be altered by the influence of some other substance, which in view of the pronounced changes, especially in the uterus, might have a more local effect on this organ. Studies are in progress to check the tenability of these suggestions.

The changes in the neuronal noradrenaline level during pregnancy and corresponding hormonal treatment do not seem to involve all sympathetically innervated organs but to be restricted to specific parts of the female reproductive tract (see also Sjöberg 1967). It should be recalled that certain parts of the female genital tract receive their sympathetic innervation by way of "short" adrenergic neurons (Owman *et al.* 1966), which have been shown to be functionally different from ordinary "long" adrenergic neurons (Euler and Lishajko 1966, Stjärne and Lishajko 1966, Owman and Sjöberg 1967, Sjöstrand and Swedin 1967). It is tempting to assume that the changes found in specific parts of the genital tract during certain stages of pregnancy and hormonal treatment (see also Sjöberg 1967) might be a further functional parameter distinguishing "short" adrenergic neurons from "long" adrenergic neurons. But it is still difficult to produce substantiating evidence for such an assumption because the changes occurring during pregnancy in oh... both an increase and a reduction in the transmitter level, besides which in some regions of the genital tract both types of neurons participate in the innervation.

This investigation was supported by grants from the Association for the Aid of Crippled Children, New York, the Swedish Medical Research Council (projects No. B67-12X-712-0 and B67-14X-56-03A), and the Medical Faculty of the University of Lund.

References

ALM, P., L. CEGRELL, B. EHINGER and B. FALCK, Remarkable adrenergic nerves in the exocrine pancreas. *Z. Zellforsch.* 1967. In press.

BERTLER, Å., A. CARLSSON, E. ROSENGREN and B. WALDECK, A method for the fluorimetric determination of adrenaline, noradrenaline, and dopamine in tissues. *K. fysiogr. Sällsk. Lund. Förh.* 1958, 28, 121—123.

CHA, K.-S., W.-C. LEE, A. RUDZIK and J. W. MILLER, A comparison of catecholamine concentration of uteri from several species and the alterations which occur during pregnancy. *J. Pharmacol.* 1965, 148, 9—13.

CORRODI, H. and N.-A. HILLARP, Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 1. Identifizierung der fluoreszierenden Produkte aus Modellversuchen mit 6,7-Dimethoxyisochinolinderivaten und Formaldehyd. *Helv. chim. Acta* 1963, 46, 2425—2430.

CORRODI, H. and N.-A. HILLARP, Fluoreszenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 2. Identifizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd. *Helv. chim. Acta* 1964, 47, 911—918.

EULER, U. S. VON and F. LISHAJKO, A specific kind of noradrenaline granules in the vesicular gland and the vas deferens of the bull. *Life Sci.* 1966, 5, 687—691.

FALCK, B., Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. *Acta physiol. scand.* 1962, 56, Suppl. 197, 1—25.

FALCK, B., N.-Å. HILLARP, G. THIEME and A. TORP, Fluorescence of catechol amines and related compounds condensed with formaldehyde. *J. Histochem. Cytochem.* 1962, 10, 348—354.

FALCK, B. and CH. OWMAN, A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta Univ. Lund.* II 1965, 7, 1—23.

HÄGGENDAL, J., An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues. *Acta physiol. scand.* 1963, 59, 242—254.

MILLER, M. D. and J. M. MARSHALL, Uterine response to nerve stimulation; relation to hormonal state and catecholamines. *Amer. J. Physiol.* 1965, 209, 859—865.

OWMAN, CH., On the significance of the 5-hydroxytryptamine stores in pineal gland. *Advanc. Pharmacol.* 1967. In press.

OWMAN, CH., E. ROSENGREN and N.-O. SJÖBERG, Origin of the adrenergic innervation to the female genital tract of the rabbit. *Life Sci.* 1966, 5, 1389—1396.

OWMAN, CH. and N.-O. SJÖBERG, Adrenergic nerves in the female genital tract of the rabbit. With remarks on cholinesterase-containing structures. *Z. Zellforsch.* 1966, 74, 182—197.

OWMAN, CH. and N.-O. SJÖBERG, Difference in rate of depletion and recovery of noradrenaline in "short" and "long" sympathetic neurons after reserpine treatment. *Life Sci.* 1967, 6, 2549—2556.

RUDZIK, A. and J. W. MILLER, The effect of altering the catecholamine content of the uterus on the rate of contractions and the sensitivity of the myometrium to relaxin. *J. Pharmacol.* 1962, 138, 88—95.

SJÖBERG, N.-O., Unpublished observations, 1967.

SJÖSTRAND, N. O. and G. SWEDIN, Effect of reserpine on the noradrenaline content of the vas deferens and seminal vesicle compared with the submaxillary gland and the heart of the rat. *Acta physiol. scand.* 1968, 72, 370—377.

STJÄRNE, L. and F. LISHAJKO, Comparison of spontaneous loss of catecholamines and ATP in vitro from isolated bovine adrenomedullary, vesicular gland, vas deferens, and splenic nerve granules. *J. Neurochem.* 1966, 13, 1213—1216.

Acta physiol. scand. 1968. 72. 425—432

From the Department of Pharmacology University of Umeå, Sweden

Further Observations on the Inhibition of Histamine Release by 2 Deoxyglucose

By

NIRMAL CHAKRAVARTY

Received 23 Juli 1967

Abstract

CHAKRAVARTY N. *Further observations on the inhibition of histamine release by 2-deoxyglucose.* Acta physiol. scand. 1968. 72 425—432.

The effect of 2-deoxyglucose (2 DG) on histamine release from rat mast cells induced by compound 48/80 and antigen-antibody reaction has been studied. In glucose-free oxygenated medium 2 DG has no effect on compound 48/80-induced histamine release and inhibits anaphylactic release only in high concentrations. Cyanide inhibition of both compound 48/80-induced and anaphylactic histamine release is however potentiated by low concentration (5 mM) of 2 DG. When glucose is used to restore histamine release blocked by cyanide, 2-DG (5 mM) markedly inhibits the release. Iodoacetate, fluoride and oxamate have similar blocking effect on the glucose-sustained histamine release in presence of cyanide. The findings are explained on the basis of a predominant inhibitory effect of 2-DG on the glycolytic pathway in rat mast cells.

As reported previously (Chakravarty 1962, Yamasaki and Endo 1963) anaphylactic histamine release from guinea pig lung is inhibited by low concentrations of 2-deoxyglucose (2 DG). The inhibition could be demonstrated both in presence of oxygen and in an anaerobic medium supplied with glucose. In contrast 2 DG was found, in a preliminary study (Chakravarty 1962), to be ineffective in blocking histamine release by compound 48/80 from minced rat lung in an aerobic milieu. In vivo 2 DG was likewise unable to block compound 48/80-induced edema in rats (Goth 1959). Since the mechanism of compound 48/80-induced histamine release in rat is very similar to anaphylactic histamine release in guinea pig (Chakravarty, Högberg and Uvnäs 1959, Chakravarty 1960) the difference in the effect of 2 DG in the two species required clarification. It is shown here that, under suitable experimental conditions, 2 DG inhibits histamine release from rat mast cells induced by compound 48/80 and antigen-antibody reaction, and the difference in the 2 DG effect on histamine release from rat and guinea pig tissues may be explained within the general frame of metabolic inhibition caused by 2 DG in different tissues

Material and method

The effect of 2 DG on histamine release from rat peritoneal mast cells was studied using mixed cell suspensions from Sprague Dawley or Wistar rats (♂ or ♀ 250—500 g). Sensitized mast cells were obtained as described previously (Chakravarty 1967).

8 ml Krebs-Ringer solution with phosphate buffer of the same composition as used previously (Chakravarty 1962) containing 50 µg/ml heparin were injected into the peritoneal cavity of the rat and the mixed cell suspension collected after massaging the abdomen for 90 sec. The cell suspension was diluted to about 25 ml with Krebs-Ringer solution containing in addition 1 mg/ml human serum albumin (pH 7.0—7.1) and the centrifuged deposit resuspended in the same solution. For antigen-induced histamine release cells were usually pooled from 2 sensitized rats for each experiment. For compound 48/80-induced release cells from one rat was used for each experiment. The cell suspension was divided into 6—10 samples. Samples treated with 2 DG or other inhibitors were incubated with the inhibitors for 10 min before adding the histamine releasing agents, compound 48/80 (0.25—1 µg/ml) or antigen (0.25—0.5 mg/ml egg albumin). Control samples without releasers and samples with the releasing agents alone were incubated for the same length of time. Incubation was carried out in 10 ml centrifuge tubes at 37° C under gentle shaking. After 5—10 min incubation with the releasers the samples were chilled to 0—4° C and centrifuged at the same temperature. The supernatant solution was collected for determining the released histamine and the cell deposit was boiled for 3 min in 0.5 ml 0.9 per cent NaCl. After cooling Tyrode solution was added and the sample centrifuged again. This supernatant solution was collected for the residual histamine content. In experiments with sodium fluoride calcium was omitted from the solution used for the cell suspension. All solutions containing inhibitors were adjusted to the same pH as the suspending medium.

Histamine was assayed by the biological method on guinea pig ileum. For testing samples containing inhibitors they were added in equal amounts to the standards. Samples containing egg albumin were boiled to precipitate the protein and prevent bubbling during the histamine test. The spontaneous histamine release (about 1 per cent) has been deducted from the values presented.

Compound 48/80 was obtained through the courtesy of AB Leo, Hälsingborg, Sweden. 2-DG (2-deoxy-d-glucose) was purchased from Sigma Chemical Company.

Results

Effect of 2 DG on histamine release in an aerobic medium

As seen in Fig. 1 2 DG even in high concentrations did not inhibit histamine release induced by compound 48/80. In contrast anaphylactic histamine release was inhibited by 2 DG although relatively high concentrations were required. 20—80 mM caused between 30 and 55 per cent inhibition of histamine release induced by antigen-antibody reaction. If this effect on histamine release is due to an inhibition

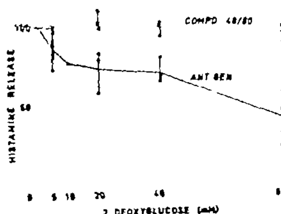


Fig. 1. Anaphylactic (●) and compound 48/80-induced (○) histamine release from Wistar rat mast cells. 100 represents histamine release in the absence of 2 DG (31—67 per cent with antigen and 60—91 per cent with compound 48/80). The points for the same 2 DG concentration represent different experiments. The result was essentially the same both for anaphylactic and compound 48/80-induced histamine release from Sprague-Dawley rat mast cells.

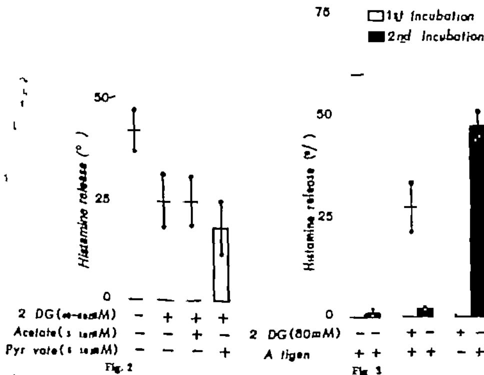


Fig. 2. The figure show the ineffectiveness of acetat and pyruv te to reverse the 2 DG inhibition of anaphylactic histamine release from Wistar rat mast cells. Acetate and pyruvate (sodium salts) were added at the same time as 2 DG. Values from tw experiments are show 40 mM 2 DG 5 mM acetate and 5 mM pyruvate were used for 0 60 mM 2 DG 10 mM acetate and 10 mM pyruvate for

Fig 3 The figure show the desensitization of Wistar rat mast cells after the first incubation with antigen in presence of 2-DG. The cells were washed between the first and the second incubation. No glucose was used for the first incubation For the second incubation 20 mM glucose as added to the medium in 1 expt. (O) and no glucose was added to the other ( ) The percentage release both for the first and second incubation relate to the total histamine content of the samples prior to the release.

of oxidati e hexose metabolism it might be reversed by acetate or pyruvate as in guinea pig tissue (Chakravarty 1962) Fig 2 shows however that the 2 DG inhibition of histamine release from rat mast cells persisted even when acetate or pyruvate was supplied to the medium.

The possibility that DG might inhibit anaphylactic histamine release from rat mast cells by different mechanism was in estigated If 2 DG inhibited anaph lactic release in the same way as suggested for dextran-induced histamine release—by block ing the dextran receptors (Goth 1966)—this would imply an inhib tion of antigen-antibody reaction by 2 DG The experiments illustrated in Fig 3 show that the mast cells even after the meager histamine release in presence of DG remained desensitized and the response to the second dose of antigen in the bsence of 2 DG was negligible. This suggests that 2 DG did not prevent the antigen-antibody re action during the first incubation.

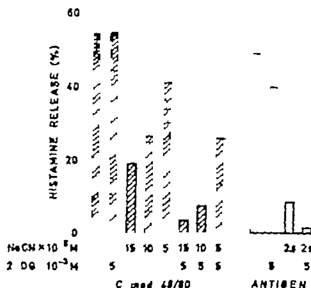


Fig. 4 The potentiation by DG of cyanide inhibition of anaphylactic histamine elease from Wistar rat mast cells and of compound 48/80-induced release from Sprague-D wley rat mast ells.

Potentiat on of cyanide nh bition of histamine release by ? DG

Although 5 mM 2 DG by itself had little or no effect on anaphylactic or compound 48 80-induced histamine release from rat mast cells it potentiated the inhibition caused by cyanide. This was characteristically demonstrated—as sho n in Fig ing a concentration of cyanide which ga e a moderate inhibition. When the nide inhibition of histamine release was more pronounced the release was usually letely blocked by adding 5 mM 2 DG to NaCN

Effect of 2 DG on the re toration by glucose of cyanide nhibition of histamin release

Fig. 5 shows that the inhibition of anaphylactic and compound 48 80-induced histamine release by cyanide was largely restored by supplying glucose to the medium, and the restoration was almost completely inhibited by a low concentration (5 mM) of ? DG If this effect of 2 DG is exerted through an inhibition of the glycolytic pathwa the same result would be expected with other inhibitors of glycolysis The effects of iodoacetate fluoride and oxamate were therefore tested.

Effect of iodoac tat flu d and o amat on the glucose sustain d histam ne release in p senc f cyanid

Fig 6 shows that ? mM iodoacetate or 10 mM fluoride had very little effect on histamine release induced by compound 48/80 in an aerobic milieu. But they completely inhibited the histamine release restored by glucose in presence of cyanide 30 mM oxamate had normall no effect on histamine release but completely inhibited the glucose-supported release in presence of cyanide. The result was essentially the same in case of anaphylactic histamine release (Fig 7) The concentrations of the

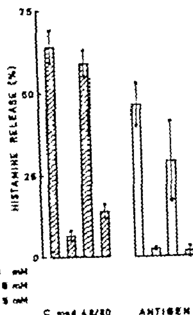


Fig. 5 Inhibition by 2-DG of glucose-dependent histamine release in presence of cyanide induced by antigen-antibody reaction (Wistar rat mast cells) and compound 48/80 (Sprague-Dawley rat mast cells) ○ and represent different experiments

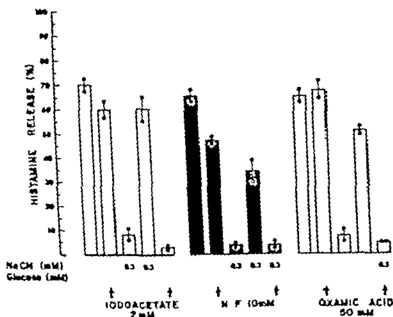


Fig. 6 Effect of inhibitors of glycolysis on glucose-dependent histamine release in presence of azide induced by compound 48/80 from Sprague-Dawley rat mast cells ○ and ● represent different experiments. Iodoacetate fluoride and oxamate sodium salts were added only for the columns indicated by the arrow

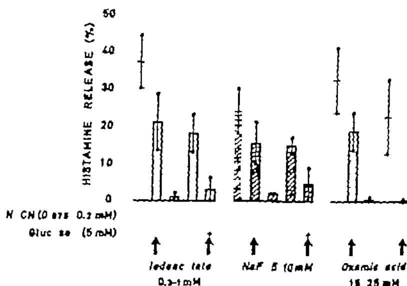


Fig. 7 Effect of inhibitors of gly olysis on glucose-dependent histamine release in presence of cyanide induced by antigen-antibody reaction from Wistar rat mast cells. Values from tw experiments. O had the lower and ● the higher of the two doses of iodoacetate fluoride and oxamat (sodium salts) which were added only for the columns indicated by the arrow

inhibitors of glycosis used here were however lower because they caused some inhibition of anaphylactic histamine release even in the absence of cyanide.

Discussion

Both anaphylactic and compound 48 80-induced histamine release in presence of oxygen were insensitive to low concentrations of 2 DG in rat in contrast to the pronounced inhibition of anaphylactic histamine release in guinea pig by 5—6 mM 2 DG (Chakravarty 1962 Yamasaki and Endo 1965). Higher concentrations of 2 DG influenced antigen- and compound 48 80-induced histamine release in rat differently. While the compound 48 80-induced release was naffected with even 20—80 mM 2 DG such concentrations caused 30 to 55 per cent inhibition of anaphylactic histamine release. This difference in the effect of DG was observed both in Sprague Dawley and Wistar rats (Fig 1). In guinea pig tissue the inhibition of anaphylactic histamine release by 2 DG was apparently caused by an inhibition of oxidative glucose metabolism and the release could be partially restored by acetate or pyru ate (Chakravarty 196. In rat anaphylaxis ? DG was much less effective and the inh bition was not reversed by acetat and pyruvate (Fig 2). This suggests a different mechanism of DG inhibition of anaphylactic histamine release in rat as compared to that in guinea pig. The effect in rat may be on aerobic glycolysis as the rat mast cells ha e a high rate of anaerobic and aerobic glycolysis (Chakravarty 1965). This explanation is supported by the observation that other

inhibitors of glycolysis also produce some inhibition of anaphylactic histamine release in rat in presence of oxygen (Fig. 7) Another possibility was considered. Histamine release induced by dextran can be blocked not only by 2 DG but also by glucose and other sugars and the mechanism of inhibition seems to be a competition with dextran for the receptor site (Dias da Silva and Lemos Fernandes 1965) The desensitization of the mast cells after contact with antigen in presence of 2 DG (Fig. 3) suggests that 2 DG does not prevent antigen-antibody reaction. The mechanism of DG inhibition of anaphylactic histamine release is thus different from that of dextran-induced histamine release.

In contrast to its ineffectiveness in compound 48 80-induced histamine release and only mild inhibiting effect in anaphylactic release in an aerobic medium, 2 DG was remarkably effective in blocking the histamine release induced compound 48 80 or antigen-antibody reaction in cyanide treated cells in presence of glucose. It has been reported previously that histamine release inhibited by lack of oxygen (Diamant 1961 1962) or by cyanide (Rothschild, Vugman and Rocha e Silva 1961) can be restored by glucose. Here it is shown that the reversal of cyanide inhibition is blocked by 2 DG The mechanism by which 2 DG blocks the glucose-supported histamine release seems to be through an inhibition of glucose transport and its glycolytic utilization. If this interpretation of 2 DG effect on the glucose-sustained histamine release in presence of cyanide is correct other inhibitors of glycolysis should have similar effect. The three inhibitors used, iodoacetate, fluorid and oxamate, inhibit 3 different enzymes of the glycolytic cycle triose phosphate dehydrogenase, enolase and lactate dehydrogenase respectively and their effect—like that of DG—was nearly a complete inhibition of the glucose-supported histamine release in presence of cyanide (Fig. 6 and 7) Histamine release in this condition thus appears to be linked with the transport and utilization of glucose by the glycolytic pathway

Although 2 DG in a low concentration did not appreciably influence histamine release from rat mast cells in an aerobic glucose-free medium, an effect could be demonstrated in presence of cyanide in the form of potentiation of the cyanide inhibition (Fig 4) This is apparently due to an inhibitory effect on endogenous hexose metabolism. It seems likely that this effect is exerted more on the glycolytic pathway It is also possible that the meager supply of ATP in presence of cyanide is trapped by 2 DG for its own phosphorylation since 2 DG is phosphorylated by hexokinase (Kipnis and Cori 1959)

The effects of DG on histamine release from rat mast cells can thus be readily explained if one assumes that the glucose analogue has in rat mast cells—as in most tissues—a strong inhibitory effect on glycolysis but only mild effect on oxidative hexose metabolism. 2 DG has indeed been shown in brain slices to be 60 times more effective in inhibiting glycolysis than glucose oxidation Woodward and Hudson 1954) Probably oxidative hexose metabolism is more sensitive to 2-deoxyglucose in guinea pig tissue—thus explaining why it inhibits histamine release in this species in the aerobic medium in much lower concentrations

It has been observed before that histamine release induced in rat by antigen-antibody reaction and compound 48/80 has a similar mechanism (Chakravarty 1960, Mota and Ishii 1960) although some differences have also been observed (Halpern *et al.* 1964). The present findings are consistent with a general mechanism of histamine release common to both antigen-antibody reaction and compound 48/80 but some inhibitors seem to have a differential effect. 2 DG in high doses gives some inhibition of anaphylactic histamine release while it is ineffective against compound 48/80-induced release. This may be due to an effect of 2 DG on aerobic glycolysis because anaphylactic histamine release appears to be more *sensitive* to inhibition of aerobic glycolysis than compound 48/80-induced release (Fig. 1, 6, 7).

This work was supported by the Swedish Medical Research Council (Project No. K67-12X-532-03).

References

CHAKRAVARTY, N., The mechanism of histamine release in anaphylactic reaction in guinea pig and rat. *Acta physiol. scand.* 1960. *48.* 146—166.

CHAKRAVARTY, N. Inhibition of anaphylactic histamine release by 2-deoxyglucose. *Nature* (Lond.) 1962. *194.* 1182—1184.

CHAKRAVARTY, N. Glycolysis in rat peritoneal mast cells. *J. cell Biol.* 1965. *25.* 123—126.

CHAKRAVARTY, N. Respiration of rat peritoneal mast cells during histamine release induced by antigen-antibody reaction. *Exp. Cell Res.* 1967. In press.

CHAKRAVARTY, N., B. HOGBERG and B. UVNÄS, Mechanism of the release by compound 48/80 of histamine and of a lipid-soluble smooth muscle stimulating principle ("SRS"). *Acta physiol. scand.* 1959. *45.* 255—270.

DIAMANT, B. Histamine release elicited by extracts from *Ascaris suis* — influence of oxygen lack and glucose. *Acta physiol. scand.* 1961. *52.* 8—22.

DIAMANT, B. *Betydelsen av glykolys och andning för histaminfrisättning och mastcellsdegranulering.* Thesis, Karolinska Institute, Stockholm 1962.

DIAS DE SILVA, W. and A. D. LEMOS FERNANDES, Study of the mechanism of inhibition produced by hexoses on histamine release activity of dextran. *Experientia* (Basel) 1965. *21.* 96—97.

GOTH, A. Inhibition of anaphylactoid edema in the rat by 2-deoxyglucose. *Amer. J. Physiol.* 1959. *197.* 1056—1058.

GOTH, A. *Mechanisms of histamine release by polymeric compounds.* Wenner-Gren Center International Symposium series on Mechanisms of Release of Biogenic Amines. 1966. *5.* 371—378.

HALPERN, B. N., M. LIACOPOULOS-BRIOT, N. SPECTOR, T. NEVEU, M. F. PERRAMANT and A. BEN-KELLEC, Méchanismes de libération de l'histamine et de la 5-hydroxytryptamine au cours de la réaction anaphylactique et sous l'action de substances histamino-libératrices (S.H.L.) chez le rat. *Arch. int. Pharmacodyn.* 1964. *147.* 431—449.

KIPNIS, D. M. and C. F. CORI, Studies of tissue permeability. V. The penetration and phosphorylation of 2-deoxyglucose in the rat diaphragm. *J. biol. Chem.* 1959. *234.* 171—177.

MOTA, I. and T. ISHII, Inhibition of mast cell disruption and histamine release in rat anaphylaxis in vitro. Comparison with compound 48/80. *Brit. J. Pharmacol.* 1960. *15.* 82—87.

ROTHSCHILD, A. M., I. VUGMAN and M. ROCHA E SILVA, Metabolic studies on the release of histamine by compound 48/80 in the rat diaphragm. *Biochem. Pharmacol.* 1961. *7.* 248—255.

WOODWARD, G. E. and M. T. HUDSON, The effect of 2-deoxyglucose on glycolysis and respiration of tumor and normal tissues. *Cancer Res.* 1954. *14.* 599—605.

YAMASAKI, H. and K. ENDO, Effect of 2-deoxyglucose, phlorizin, insulin and ouabain on glucose-dependent anaphylactic histamine release from guinea-pig lung. *Jap. J. Pharmacol.* 1965. *15.* 48—56.

Acta physiol. scand. 1968. 72. 433—440

From the Institute for Experimental Research in Surgery Copenhagen, Denmark

The Effect of Synthetic Secretin on the External Pancreatic Secretion in Dogs

By

F W Henriksen

Received 25 July 1967

Abstract

Henriksen, F W *The effect of synthetic secretin on the external pancreatic secretion in d[illegible]g[illegible]* Acta physiol. scand. 1968. 7[illegible] 433—440.

The effect of synthetic secretin on the external pancreatic secretion was investigated in three dogs with Thomas fistulas and gastrostomies. The dose-response curv[illegible] was determined in two dogs. The maximal secretory capacity equalled that obtained with other secretin preparations. Supramaximal doses of synthetic secretin did not depress the secretion of fluid or bicarbonate. A short-lasting increase in the protein secretion after injection of synthetic secretin is explained as [illegible] "[illegible]ash-out" phenomenon.

Since secretin was discovered by Bayliss and Starling (190[illegible]) the effect on the external pancreatic secretion of this hormone has been extensively studied. However the secretin preparations used ha[illegible]e not been purified to a uniform degree, and all preparations have contained pancreozymin, which may explain some of the contradictory results obtained.

The work of Jorpes and Mutt (Mutt 1959, Jorpes and Mutt 1961 Jorpes *et al.* 1962, Mutt *et al.* 1965 Mutt and Jorpes 1966, 1967) to elucidate the structure of the secretin has recently resulted in a synthetic secretin. (Bodanszky [illegible]*t al.* 1966 a, b, Bodanszky and Williams 1967)

This study presents the results obtained with synthetic secretin in dogs.

Materials and methods

Three mongrel dogs were used (weight 19.5—26.0 kg) After ligation of the accessory pancreatic duct [illegible] modified Thomas cannula (1941) was inserted into the duodenum [illegible] the main pancreatic duct. The same type of cannula was inserted at the most deper[illegible] of the stomach. The dogs were used for experiments after [illegible] recovery period of at [illegible] weeks.

During the experiments the dogs were standing in [illegible] sling harness. The gastric [illegible] opened to prevent gastric secretion from entering the duodenum. A glass catheter [illegible] glass syringe by means of [illegible] short polyvinylchloride tube was inserted into [illegible] creatic duct through the open duodenal fistula. The pendant plunger of the [illegible] slight negative pressure. The dead space of the collecting system was 0.4 ml.

It has been observed before that histamine release induced in rat by antigen-antibody reaction and compound 48/80 has a similar mechanism (Chakravarty 1960, Mota and Ishii 1960) although some differences have also been observed (Halpern *et al.* 1964). The present findings are consistent with a general mechanism of histamine release common to both antigen-antibody reaction and compound 48/80 but some inhibitors seem to have a differential effect. 2-DG in high doses gives some inhibition of anaphylactic histamine release while it is ineffective against compound 48/80-induced release. This may be due to an effect of 2-DG on aerobic glycolysis because anaphylactic histamine release appears to be more sensitive to inhibition of aerobic glycolysis than compound 48/80-induced release (Fig. 1, 6, 7).

This work was supported by the Swedish Medical Research Council (Project No. K67-13X-552-03).

References

Chakravarty, N. The mechanism of histamine release in anaphylactic reaction in guinea-pig and rat. *Acta physiol. scand.* 1960. *48.* 146—166.

Chakravarty, N. Inhibition of anaphylactic histamine release by 2-deoxyglucose. *Nature* (Lond.) 196[illegible]. *194.* 1182—1184.

Chakravarty, N. Glycolysis in rat peritoneal mast cells. *J. Cell Biol.* 1965. *25.* 123—126.

Chakravarty, N. Respiration of rat peritoneal mast cells during histamine release induced by antigen-antibody reaction. *Exp. Cell Res.* 1967. In press.

Chakravarty, N., B. Högberg and B. Uvnäs, Mechanism of the release by compound 48/80 of histamine and of a lipid-soluble smooth muscle stimulating principle ("SRS"). *Acta physiol. scand.* 1959. *45.* 255—270.

[illegible], B. Histamine release elicited by extracts from *Ascaris suum* — influence of oxygen lack and glucose. *Acta physiol. scand.* 1961. *52.* 8—22.

Diamant, B., *Betydelsen av glykolys vid histaminfrisättning och mastcellsdegranulering.* Thesis. Karolinska Instit. Stockholm 1962.

Dias da Silva, W. and A. D. Lemos Fernandes, Study of the mechanism of inhibition produced by hexoses on histamine release activity of dextran. *Experientia* (Basel) 1965. *21.* 96—97.

Goth, A. Inhibition of anaphylactoid edema in the rat by 2-deoxyglucose. *Amer. J. Physiol.* 1959. *197.* 1056—1058.

Goth, A. *Mechanisms of histamine release by polymeric compounds.* Wenner-Gren Center International Symposium series on Mechanisms of Release of Biogenic Amines. 1966. *5.* 371—3[illegible]8.

Halpern, B. N., M. Liacopoulos-Briot, A. Simoas, T. Neveu, M. F. Perram and A. Ba-nellec, Mechanismes de libération de l'histamine et de la 5-hydroxytryptamine au cours de la réaction anaphylactique et sous l'action de substances histamino-libératrices (S.H.L.) chez le rat. *Arch. Int. Pharmacodyn.* 1964. *147.* 431—449.

Kipnis, D. M. and C. F. Cori, Studies of tissue permeability. V. The penetration and phosphorylation of 2-deoxyglucose in the rat diaphragm. *J. biol. Chem.* 1959. *234.* 171—177.

Mota, I. and T. Ishii, Inhibition of mast cell disruption and histamine release in rat anaphylaxis in vitro. Comparison with compound 48/80. *Brit. J. Pharmacol.* 1960. *15.* 82—87.

Rothschild, A. M., [illegible] and M. Rocha e Silva, Metabolic studies on the release of histamine by compound 48/80 in the rat diaphragm. *Biochem. Pharmacol.* 1961. *7.* 248—253.

Woodward, G. E. and M. T. Hudson, The effect of 2-deoxyglucose on glycolysis and respiration of tumor and normal tissues. *Cancer Res.* 1954. *14.* 599—605.

Yamasaki, H. and K. Endo, Effect of 2-deoxyglucose, phlorizin, insulin and ouabain on glucose-dependent anaphylactic histamine release from guinea-pig lung. *Jap. J. Pharmacol.* 1965. *15.* 48—56.

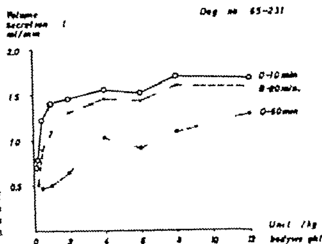


Fig. 2. Relation between dose of secretin and pancreatic olume secretion rate, calculated for the periods 0—10 min, 0—20 min and 0—60 min.

and 0—60 min. As evaluated from the curve 0—10 min, th maximal secretion was obtained with 8 U kg b.w In the other dog it was obtained with 1 U kg b.w An increase in dose with 50 and 800 per cent respectively did not change the response.

S cretion of bicarbonat

The bicarbonate concentration in the juice increased immediately after the injec tion of secretin. In the period 0—2 min it exceeded 130 meq l in all experiments. The period elapsing to obtain the peak concentration varied from experiment to experiment, ranging from 3 to 11 min after stimulation, w thout relation to the dose of secretin used.

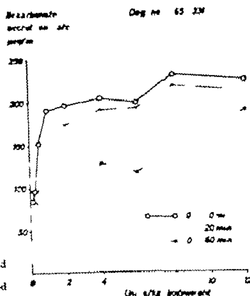


Fig. 3. Relation bet een dose of secretin and pancreatic bicarbonate secretion rate calcula ed for the periods 0—10 min, 0—20 min and 0—60 min.

Except for small amounts of w ter the dogs were fasted for 18 hours befor the experiment. The fasting pancreatic secretion as collected for at least tw periods of 15 min. After stimulation the secretion was collected until the effect of secretin had subsided. The collecting periods were 2 min for the first 20 min after stimulation, and afterwards 10 min.

The synthetic secretin preparation used had the composition of amino acids as described by Mutt et al. (1965) and Bodanszky et al. (1966). It was batch M. B. IV no. 132 Squibb Institute for Medical Research, New Brunswick, N. J., U.S.A., and it contained 900 clinical Units/mg (1 clinical unit = 20 Hammarsten cat units). The secretin was dissol ed in isotonic saline to a concentration of 10 U/ml immediately before use, and injected in a peripheral vein either as single intravenous injection or as continuously intravenous infusion. The single injections were made within 10—15 sec, irrespectiv of the dose. The different doses given to each dog were injected in randomized order. No more than three injections were given each day of experiment. The interval between injections was at least 80 min, and for doses above 1 U/kg b.w. at least 120 min (Henriksen 1966). The infusion was continued for 160 min. As the supplies of the synthetic secretin were limited, only one experiment was made at each dose level in each dog.

The volume of the pancreatic secretion and the bicarbonate and protein concentration in the samples were measured. The concentration of total CO_2 was determined according to Van Slyke's manometric method (1924). The concentration of protein was estimated spectrophotometrically at 280 nm after appropriate dilution of the samples with Sørensen phosphat buffer pH 6.8. The extinction alues were converted to concentrations of protein by means of factor empirically determined through Kjeldahl analysis on pooled canine pancreatic juice.

Results

Secretion of fluid

The secretion of fluid increased within 1 min after the injection of the secretin preparation. Regardless of the secretin dose used, the peak secretion rate of fluid in each experiment occurred within the first or second 2 min period. After a high dose of secretin (8 U/kg b.w.) the curve shows an almost linear decline. The curve obtained smaller doses was more or less S-shaped, but all had an almost rectilinear por (Fig. 1).

Dose-response curves for dog no. 65—231 are shown in Fig. 2. The secreted volumes are expressed as the mean secretion rate in the periods 0—10 min, 0—20 min

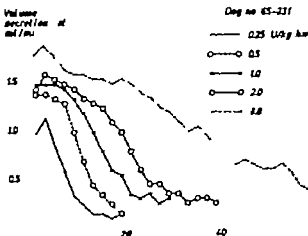


Fig. 1. Relation between pancreatic olume secretion rate and time after intravenous injection of synthetic secretin.

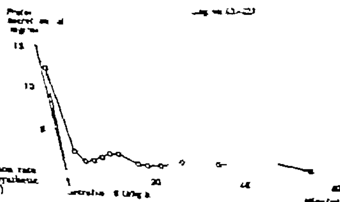


Fig. 8. The protein secretion rate after injection of synthetic secretin (6 Units/kg b.w.)

TABLE I. Protein secretion rate (mg/min) in the period 0—10 min after injection of synthetic secretin

Dose of secretin Units/kg b w	Dog no.	
	65—231	66—177
0.125	3.5	4.8
0 25	3.2	3.4
0.5	3.6	3.9
1.0	6.8	9.3
2 0	3.2	5.3
4.0	2.8	4.1
6.0	3 7	5.1
8.0	4.5	2.7
12.0	4.4	—

superimposed as rapid injections in another vein. The protein secretion rate was determined in 10 min periods, except immediately after the superimposed secretin stimulation, when 2 min samples were used (Fig. 7) No significant deviation from non-stimulated protein secretion rate was observed after the smaller doses of secretin. However significant increase in protein secretion rate was seen after injection of 4 U kg b w in the period 0—2 min., after which it subsided to a basal level.

Discussion

The dose of synthetic secretin required to obtain maximal pancreatic response differed widely in the two dogs investigated. This phenomenon is known from investigations with highly purified secretin (Baron et al. 1963 and Hansky et al. 1964). The dogs used in the present investigation had their maximal secretory capacity determined in previous experiments. In dog no. 65—231 and 66—177 Secretin Boots was



FIG. 4

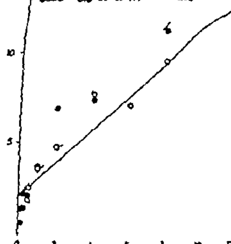


Fig. 5

Fig. 4 Relation between the logarithm of the dose of secretin and total bicarbonate output.
Fig. 5 Relation between the dose of secretin and total bicarbonate output.

Dose-response curves for bicarbonate secretion (Fig 3) are almost identical to the curves of Fig. 2. In the two dogs the level of maximal secretory rate was obtained with 8 and 1 U kg b.w respectively Increasing the doses with 50 per cen and 800 per cent respectively did not change the response

Fig 4 and 5 present the relation between the total amount of bicarbon secreted and the dose of secretin in two dogs. The approximation to a straight relation was equally good when plotted on a semilogarithmic scale (Fig 4) an a linear scale (Fig 5)

Secretion of protein

After injection of synthetic secretin the protein secretion rate increased non-stimulated level within 0– 2 min. (Fig. 6) In one case this increase 0—4 min. There was no relation however between the secretion rate obtained and the dose of secretin used (Table I) The increase in prot might be explained as a "wash-out" phenomenon. To investigate this stimulated with a continuous infusion of secretin and different doses of

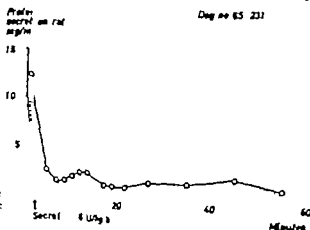


Fig 6 The protein secretion rate after injection of synthetic secretin (6 Units/kg b w)

TABLE I. Protein secretion rate (mg/min) in the period 0—10 min after injection of synthetic secretin

Dose of secretin Units/kg b w	Dog no 65—231	66—177
0.125	3 3	4.8
0 25	3 2	3 4
0 5	3 6	3 9
1 0	6 8	9.9
2 0	3 2	3 3
4.0	2 8	4.1
6.0	3 7	5 1
8 0	4	2.7
12 0	4 4	—

superimposed as rapid injections in another vein. The protein secretion ... determined in 10 min periods except immediately after the superimposed ... stimulation, when 2 min samples were used (Fig. 7). No significant de... non-stimulated protein secretion rate was observed after the smaller doses of ... However a significant increase in protein secretion rate was seen after ... 4 U kg b w in the period 0 ... min after which it subsided to a basal l...

Discussion

The dose of ... hetic sec... required to obtain maximal pancrea... fered widel... the ... do... estigated. This phe... is know... tions with highly purified sec... n (Baron et al. ...) and Hanak... dogs used ... th present investigation had their maximal secretor... mined in pr... periments. In dog no. 65—231 and 66—177

bicarb... secretion ... ecapepid...

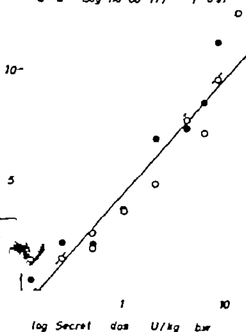


Fig 4

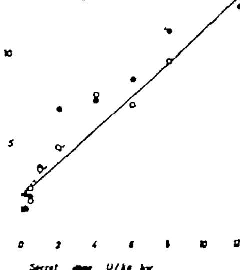


Fig 5

Fig. 4 Relation between the logarithm of the dose of secretin and total bicarbonate output.
Fig. 5. Relation between the dose of secretin and total bicarbonate output.

Dose-response curves for bicarbonate secretion (Fig. 3) are almost identical to the curves of Fig 2. In the two dogs the level of maximal secretory rate was obtained with 8 and 1 U/kg b.w respectively. Increasing the doses with 50 per cent and 800 per cent respectively did not change the response.

Fig 4 and 5 present the relation between the total amount of bicarbonate secreted and the dose of secretin in two dogs. The approximation to a straight line relation was equally good when plotted on a semilogarithmic scale (Fig. 4) and on a linear scale (Fig. 5).

Secretion of protein

After injection of synthetic secretin the protein secretion rate increased above the non-stimulated level within 0—2 min. (Fig 6) In one case this increase lasted for 0—4 min. There was no relation however between the secretion rate of protein obtained and the dose of secretin used (Table I). The increase in protein secretion might be explained as a "wash-out" phenomenon. To investigate this, a dog was stimulated with a continuous infusion of secretin and different doses of secretin were

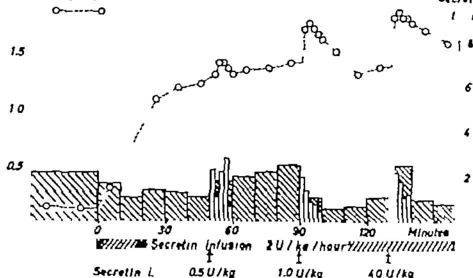


Fig. 7 The pancreatic secretion of protein after single jections of synthetic secretin, super on constant stimulation w th synthetic secretin
hatched columns indicate the mean protein secretion rate in periods of 10 min the columns in periods of 2 min.

and in dog no. 65—231 a highly purified secretin was used too (G I H Research Unit, Chemistry Dept., Karolinska Institutet, Stockholm) The maximal secretory level obtained with synthetic secretin did not differ from the one obtained with the secretin preparations mentioned. The dose of secretin (Units kg b w needed to obtain maximal secretion, however varied from one preparation to the other (Table II) This emphasizes the fact that a standardization of the secretin has not been possible.

No depressing effect on the olume or bicarbonate secretion was observed after supramaximal doses of synthetic secretin, indicating that the depressing effect on the

Table II. Maximal pancreatic secretory level btained with differen secretin preparations

Dog no.	Secretin preparation	Dose U kg b w	Volume secretion rate ml min	Bicarbonate secretion rate μeq min
65—231	Boots	8	1 75	219
	Highly purified (Jorpes-Mutt)	4	1 74	233
	Synthetic	8	1 70	232
66—177	Boots	10	1 98	250
	Synthetic	1	1 85	243

bicarbonate secretion observed by Baron *et al.* (1963) with Secretin Vitrum in supramaximal doses, seems to be explained by the effect of other substances than secretin, present in their secretin preparation.

Considering the volume secretion in the periods 0—10 and 0—20 min (Fig. 2) it is obvious that no linear relation exists between the secretion and the dose of secretin, except in a very narrow dose-range. When plotted on a semilogarithmic scale no linear relation, including the whole range, was seen. The volume secretion in the period 0—60 min was approximated to a straight line in a linear scale, but all the same it appeared as a straight line in a semilogarithmic scale. On account of the uncertainty of the points of measurement it could not be decided whether the relation was linear or semilogarithmic.

The relation between the total amount of bicarbonate secreted and the logarithm to the dose of secretin, showed a good approximation to a straight line (Fig. 4). The stoichiometric proportionality was, however, equally good (Fig. 5).

Whether secretin affects the pancreatic secretion of enzymes has been a problem hard to approach, as the secretin hitherto available has contained some pancreozymin. An increase in the protein secretion rate was observed in the initial minutes after stimulation with synthetic secretin (Fig. 6). However, there was no relation between the protein secretion rate obtained and the dose of secretin used (Table I). When superimposing single injections of secretin on a background stimulation with secretin no increase in protein secretion rate was observed after smaller doses of secretin. A clear but short lasting increase was seen after 4 U/kg b.w. (Fig. 7) similar to the increase after a single injection (Fig. 6). If under these conditions, the increased protein secretion rate is to be explained as a "wash-out" phenomenon, the protein must be derived from resting secretory units, stimulated only by high doses of secretin.

Most of the data presented point to the conclusion that synthetic secretin does not affect the secretion of protein. It cannot be completely excluded, however, that high doses of secretin have a short-lasting effect on the protein secretion.

Prof. E. Jorpes and Dr. V. Mutt have most kindly supplied the synthetic secretin preparation. This work has been supported by grants from "Statens almindelige Videnskabsfond", "Danish foundation for the advancement of medical science" and "P. Carl Petersens foundation".

References

Baron, J. H., C. V. Perrier, H. D. Janowitz and D. A. Dreiling, Maximum alkaline (bicarbonate) output of the dog pancreas. *Amer. J. Physiol.* 1963, *204* 251—256.

Bayliss, W. M. and E. H. Starling, On the causation of the so-called "peripheral reflex secretion" of the pancreas. *Proc. roy. Soc. B* 1902, *69* 352—353.

Bodanszky, M. and N. J. Williams, Synthesis of secretin. I. The protected tetradecapeptide corresponding to sequence 14—27. *J. Amer. chem. Soc.* 1967, *89*, 685—689.

Bodanszky, M., S. D. Levine, V. Narayanan, M. A. Ondetti, M. von Saltza, J. T. Sheehan and N. J. Williams, Confirmation by synthesis of secretin sequences. IUPAC 4th Congr. of Natural Products. Stockholm. June 26th—July 2nd. 1966a. Section 2C—2.

Bodanszky, M., M. A. Ondetti, S. D. Levine, V. Narayanan, M. von Saltza, J. T. Sheehan, N. J. Williams and E. F. Sabo, Synthesis of a heptacosapeptide amide with the hormonal activity of secretin. *Chemistry and Industry* Oct. 15th, 1966b. pp. 1757—58.

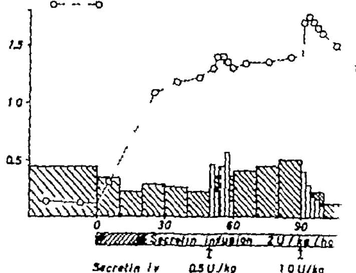


Fig. 7 The pancreatic secretion of protein afte single inj imposed on constant stimulation with synthetic secretı atched columns indicate the mean protein secr ns in periods of 2 min.

d in dog no. 65—231 a highly purified sec ɛ ı Unit, Chemistry Dept., Karolinska Institutet. ory level obtained ith synthetic secretin did not d the secretin preparations mentioned. The dose of s obtain maximal secretion, however varied from one II) This emphasizes the fact that a standardizat possible.

No depressing effect on the volume or bicarb supramaximal doses of synthetic secretin, indicating

TABLE II Maximal pancreatic secretory lev l obtained w

Dog no.	Secretin preparation	Dose U kg b.w	V sec
65—231	Boots	8	1
	Highly purified (Jorpes-Mutt)	4	1
	Synthetic	8	1
66—177	Boots	10	1
	Synthetic	1	1

Acta physiol. scand. 1968. 72. 441—455

From the Institute of Neurophysiology University of Copenhagen, Denmark

Response of Isolated Muscle Spindles to Single Transient Stretches*

By

Samia Al Azharia Jahn

Received 26 July 1967

Abstract

Jahn, Samia Al Azharia. *Response of isolated muscle spindles to single transient str tckes.* Acta physiol. scand. 1968. 72 441—455

The smallest stretch of isolated frog muscle spindles which regularly evoked an afferent discharge was 8±2 μ measured as the displacement of a graphite granule placed above the equatorial region 5—8 mm from the distal tendon of the muscle. The threshold deformation (T) was independent of an increase in the rate of deformation from 0.016×T/msec to 1.0×T/msec and of an increase in the initial elongation from 10 to 25 per cent above equilibrium length. The rate below which the spindle did not respond to stretch was 0.01×T/msec. With low rates of stretch (0.016—0.03×T/msec) the afferent response occurred before the deformation was completed and with higher rates (0.1—1.0×T/msec) after it was completed.

A second afferent response was elicited when the deformation exceeded 2 times threshold (rate of stretch 0.1—0.3×T/msec) or 3—4 times threshold (rate of stretch 0.6—2.7×T/msec). The shortest interval between two afferent responses was 2 msec.

Depending on the timing of the spontaneous afferent activity and the spontaneous intrafusal contractions the afferent response evoked by stretch was abolished or its latency was shortened or increased.

In the classical studies of amphibian muscle spindles the afferent response was lead off from the nerve of thin muscles containing few stretch receptors (Adrian and Zotterman 1926, Matthews 1931). A more detailed analysis of the response pattern was carried out by Katz (1950) on semi-isolated preparations and by Shepherd and Ottoson (1965) on isolated muscle spindles in which the extracapsular portions of the intrafusal muscle fibres were crushed. In both these series the deformation was constant and above threshold.

The response of the spindle to a deformation near threshold has so far not been studied. The aim of the study presented in this report was to investigate in isolated intact muscle spindles the influence of the size and rate of deformation on the afferent response to transient stretches. Moreover the effect was studied of spontaneous contractions of single intrafusal muscle fibres on the afferent response.

* Preliminary reports Jahn (1963) and Jahn (1966

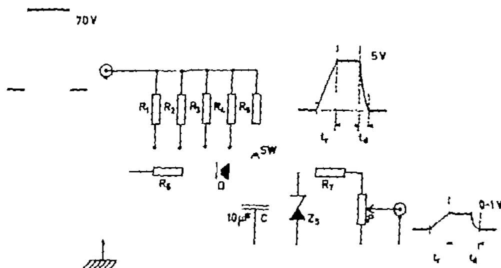


Fig. 1 Pulse shaping circuit to transform rectangular pulse to a linearly rising pulse of given rise time

R_1—R_5 resistors through which the condenser was charged t 5 V he resistor determines the rise time of stret h (t_r) 1 msec 6.8 kΩ, 100—110 msec 1 MΩ
condenser (10 μF)
Zenar diode, limiting the condenser voltage to 5 V
esistor and diod determining the decay-time (t_d)
resistor and potentiometer to ary the impulse amplitude between 0 nd 1 V
witch

Methods

P pa atio The experiments were performed on isolated muscle spindles of the m extensor longus digiti IV of *Rana t mpo aria*. Most experiments were carried ou on summer frogs; care was taken that the extensor muscle had a minimum of connectiv tissue. The spindle was isolated over length of 1—2 mm. The muscle tissue in series with the isolated portion of the muscle spindle consisted of spindles in series with the isolated one, the extracapsular portions of the intrafusal muscle fibres belonging to it and about 10 damaged extrafusal muscle fibres (Jahn 1959). The tissue parallel w th he isolated portion of the muscle spindle consisted of remnants of damaged neighbouring sp ndles in addition to fine strands of connectiv tissue around the pindle capsule and betwee the spindles. The preparation as mounted at the tw tendons as described (Jahn 1967 The muscle ut was 12 14 mm long, 15 per cent abov equilibrium length, the length t which the preparation was just taut. The equilibrium length as determined by epeated slow stretches and releases was reproducible within 6—8 per cent (Jahn unpublished). I the experiments the degree of stretch was 10—25 per cent. The mean diameter in the equatorial region of the isolated spindles was 70—80 μ and 3 intrafusal muscle fibres were seen this number was however not controlled histological sections. The proximal tendon of the extensor muscle as ied to glass rod onnected to stretch device. The initial longations were measured as described (Jahn 1967)

St et h devic Rectangular pulses (DISA Myostim were transformed in pulse shaping unit (Fig. 1) to pulses with rise times of 1 110 msec F e different rise times were studied in each experiment, 3 or 4 fast 1—10 msec) and 1 or 2 slow tret hes (20—110 msec)

The stretch was maintained for 500 msec and the ime of decay as 2 msec. The rectangular pulses were amplified and transformed to stretches as he oil of loud peaker (Fig. 2) The coil was centered around a thin-walled aluminium cone cemented to an aluminium tube sliding in tw nylon bearings. The tube was kept in longitudinal position by cotton diaphragms. One end of the aluminium tube was onnected to glass rod. The loud speaker coil and the optical feed-back system were mounted on slide and withdrawn from the experimental chamber while the preparation was transferred it.

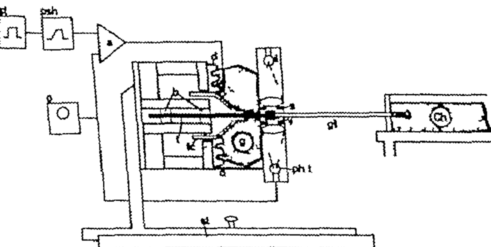


Fig 2 Schematic diagram of the stretch device and the optical feed back system
st stimulator psh pulse shaping unit, d.c. mplifier lc loudspeaker coil, cathode ray oscilloscope, t aluminum tube, gl glass rod, b bearings (nylon) d cotton diaphragm, g damping grease l light source, slit, ane, ph t photo-transistor, sl slide, ch experimental chamber

Optical f d back F g 2) The movements of the glass rod wer ontrolled photo-electricall For this purpo.e ane attached to the end of the aluminium tube moved in a beam of parallel light bet een two lenses. The ariations of light intensity were measured by photo-transistor OCP 70) and fed back to the amplifier (Fig 2) to increase the stiffness of the mechanical system. The photo-electrical signals were displayed on one beam of the oscilloscope as measur of the deformation.

Calibratio of the pindle displacement The output from the photo-transistor aried linearl with the movements of the glass rod ithin 0 5—1 5 mm. Stretches ith ise times of 1—5 msec induced oscillations of the loudspeaker coil which lasted for 2—4 msec after the transient. The moveme ts of the glass od wer damped by placing grease around the aluminium one. Thereby the ibrations wer reduced, but not entirely avoided the artefact as most pronounced hen the rise time as 1 msec (Fig. 4 and 11)

The deformation of he spindle as measu ed by placing one or tw graphite granules abov the equatorial egion of the muscle spindle 1 10 spindles the displacement of each graphite granule as studied in detail it aried linearly ith the movement of the loudspeaker coil in the range of 100— 00 Fig 5) displacements of less than 100 ere extrapolated from this relationship With rise times of 20 or 40 msec the displacement differed less than 10 per cent from the displacemen in stretches with rise times of 10 msec

T investigate hether fast stretches of 2 —5 msec ise time elicited an overshoot the time ourse of the displacemen as recorded photo-electrically i expts) Part of the equatorial region as overed by small piece of onnectiv tissue ith ll-defined border The movemen of this darker section ere followed photo-electricall and ecorded simultaneously ith the moveme of he glass od Ther was no sign of an overshoot in the initial phase of the st etch The de f rmation measured by the displacemen of graphite granule on the sur fac of he equa rt al egion depends on the length of musc in series th he equatorial region I tw F 3 ot the spindles as about 8 mm, in one third abou 3 mm

The *Ri g* d ontained 115 mM NaCl 2.7 mM KCl 1 8 mM CaCl₂, 0 2 g per liter gluco nd 3f per li er dextran The temperature of the Ringer bath as 17 20 C

Record g The me hanical ansients ere ecorded simultaneoul th he action potentials from the muscle s le on one beam of an oscilloscope Dumon 279

The amplifiers oupled they had athode follower input ith an npu impedance of 10(M) and effectiv apacity to ground of about 5 pF The lower limiting f equenc per 5 10 kc/s (3 db dow Extracellular afferent discharges and spontaneou disch ge he intrafusal muscle fibres er ecorded in the perifascicular

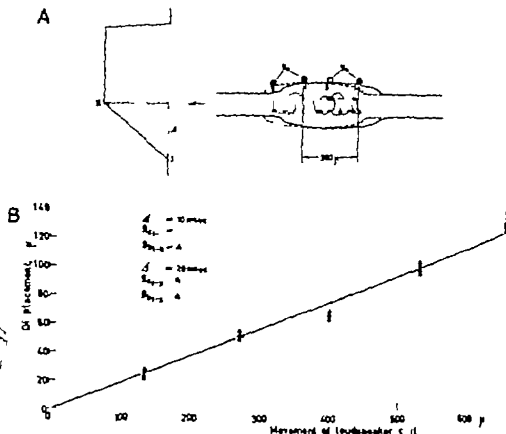


Fig. 5 Measurement of the deformation in transient stretch.
A Displacement of the two graphite granula g_1 and g_2 by transient stretch with rise time Δ
B Example of the rectilinear relationship between the movement of the loudspeaker coil and the displacement of the granules box the equatorial region of spindle subjected stretches with rise times of 10 and 20 msec and durations of 200—500 msec.

space of the equatorial region by glass capillary micro-electrodes (filled with 5M NaCl, impedance 4—8 MΩ) inserted through the capsule of the spindle (Jahn 1967

Receptor potentials could not be discriminated on account of movement artefacts.

In some experiments the artefact arising from the movement of the electrode was reduced by ming movable recording electrode. It onsisted of carrier microelectrode with broken tip (impedance 1 MΩ) which was placed in the pper end of the glass capillary of the recording electrode and sealed by rubber film (Sten-Knudsen, O personal communication)

M of latency The latency of the afferent response was measured to the peak of the initial spike, which occurred less than 1 msec after the onset of the action potential.

Results

The thr shold of formation (T)

The smallest stretch of the muscle spindle which always evoked an afferent response was denoted threshold deformation. The repeat rate of stretches was less than 1 sec. The response was considered regular when ( ) 80 per cent or more of the stretches were followed within less than 10 msec by an afferent discharge and (*ii*) the S.D.

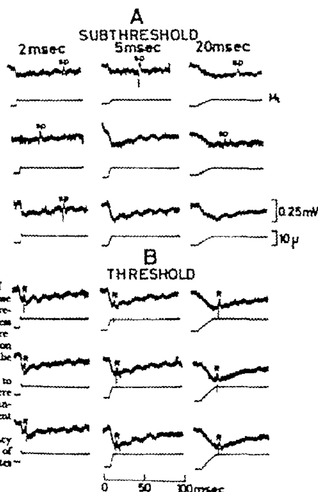


Fig. 4 The afferent response of muscle spindle to stretches with rise times of 2,5 and 20 msec. The repeat ate of the stretches as less than 1/sec. The esponses ere recorded from the equatorial region ith an initial elongation of the preparation of 20—25 per cent.
A Regula afferent responses to deformations of 3—7 μ ere absent M, mechanical transient. sp spontaneous afferent discharge.
B Responses ith egular latency elicited by deformations of 8—10 μ at slow and fast rates of stretch
R afferent response.

of the latency of the evoked discharge was less than 40 per cent of the erage latency (Fig 4B 5A and Table II) The threshold deformation evoked singl afferent discharge (Fig. 4B) Deformations 1—2 μ below threshold me ured n series with 5—8 mm muscle tissue p 3) occasionally evoked a response W th till smaller deformations the afferent discharge occurred at random (Fig 4A nd Fig 6A) For comparison of different preparations the deformation was expressed in units of threshold, since the absolut values depend on the elastic pr perties of the preparation and the length of tissue in series with the equatorial region

a. Rise t m f 2 20 mse In 23 muscle pindles the threshold def rmatio ried between 7 and 10 μ As a function of rise time the threshold deformation ried n 19 spindles by at most 1 μ in 4 by μ. The variation in threshold ncreased w th decreasing rate of deformation (Fig 5B and S.D of threshold determina ions in Table I The threshold deformation was the same whether mo able r ordinary

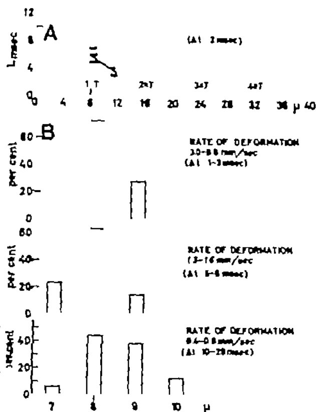


Fig. 5:
A Determination of threshold (T). L latency

deformation μ	number of stretches	number of afferent discharges
2	3	0
4	4	2
5	3	0
6	3	0
7	2	0
8 (T)	12	11

Deformations exceeding 2 T evoked responses with latency of 5 msec.
B Distribution of threshold deformations.
Rate of stretch in units of threshold deformation
above 0.3—1.0 × T/msec
middle 0.16—0.2 × T/msec
below 0.05—0.1 × T/msec.

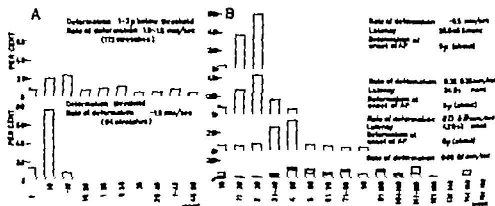


Fig. 6
A: Latency" distribution of afferent discharges after single transient stretches with a rise time of 5 msec (17 spindles)
Above The deformation was slightly below threshold. Most afferent discharges were spontaneous, some evoked responses with latencies of 6—15 msec.
Below The deformation was threshold (7—9 μ). Most afferent discharges were evoked responses with latency of 6—10 msec.
B Latency" distribution of afferent discharges after single transient stretches with deformations of 8—10 μ ("T" for stretches with rise times of 1—10 msec) and rates of deformation between 0.01 and 0.06 × T/msec. (35—54 stretches each group)
□ first of two afferent discharges (spontaneous?) within 160 msec after the onset of the stretch.
□ second of two afferent discharges (spontaneous?) during the same period of observation.

TABLE I Threshold deformation which evoked an afferent response (repeat rate below 1/sec)

	Initial elongation 10—15 per cent			Initial elongation 20—25 per cent		
rise time Δt msec	number of spindles	threshold deformation μ	SD μ	number of spindles	threshold deformation μ	S.D. μ
1				2	8.5	
1.5	2	8.5		3	8.0	
2	3	8.4	(0.5)	14	8.3	0.5
2.5—3	2	8.0		17	7.9	0.6
5	3	7.7				
6	2	8.0				
10	7	8.4	1.1	11	8.3	0.8
20	4	8.8		12	8.6	0.8

microelectrodes were inserted for recording. The average threshold deformation was 8.3 μ independent of the initial elongation (10—25 per cent) and of the rate of deformation (Table I).

When the deformation was measured as the increase in distance between two graphite granules placed on the equatorial region 300—400 μ apart, the threshold estimated by extrapolation was 0.5—1 μ (Fig. 5).

Latency. With threshold stretches the average time from the completed stretch to the afferent response ("reduced" latency L_r, Table II) was independent of rise times of 1—10 msec. With a rise time of 20 msec the "reduced" latency was 3—5 msec shorter.

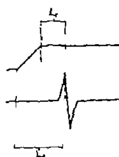
When the rise time was 1—2 msec responses occurred no less than 2 msec after completed stretch (2—8 msec, Table II); when the rise time was 5—10 msec some responses occurred at the end of the stretch (Table II). With rise times of 1—10 msec the "reduced" latency decreased with increasing initial elongation and with a rise time of 20 msec it was independent of the initial length (Table II).

b. Rise time of 20—100 msec. With slow stretches it was difficult to determine the threshold deformation, since spontaneous afferent discharges (frequency at an initial elongation of 20—25 per cent about 10/sec) interfered with the true responses. If one assumes that the spontaneous discharges are distributed randomly, the percentage of true responses and their latencies can be estimated from the histogram of latencies (Fig. 6). The mean latency of responses to stretches with rates below 0.05 × T msec was shorter than the rise time (Table III). The S.D. of the latency was doubled when the rate of stretch was decreased from 0.04 × T msec to 0.016 × T msec (Table III).

At the onset of the afferent action potential the deformation had reached 6—9 μ (Table III). In fact the threshold deformation was smaller on account of the

TABLE II Latency of the afferent response to threshold deformations

rise time Δt msec	Initial elongation 10—15 per cent: number of spindles	total latency (L_d) msec	reduced latency (L_r) msec	S.D msec	Initial elongation 20—25 per cent: number of spindles	total latency (L_d) msec	reduced latency (L_r) msec	S.D msec	Difference between latencies at the t elongations msec
1					2 (10)	4.2±0.3	3.2±0.3	0.9	
1.5	2 (14)	7.7±0.7	6.2±0.7	2.7	3 (9)	5.0±0.3	3.5±0.3	0.9	2.7±0.8
2	5 (18)	8.3±0.4	6.3±0.4	1.6	14 (92)	6.0±0.2	4.0±0.2	1.9	2.3±0.4
2.5—3	2 (8)	9.7±0.5	6.7±0.5	1.5					
5	3 (10)	11.5±0.9	6.5±0.9	2.8	17 (94)	8.0±0.3	3.0±0.3	3.2	3.5±0.9
6	2 (10)	13.1±0.8	7.1±0.8	2.7					
10	7 (35)	15.7±0.5	5.7±0.5	3.2	11 (40)	13.0±0.5	3.0±0.5	3.4	2.7±0.7
20	4 (18)	21.4±1.4	1.4±1.4	3.9	12 (55)	20.0±0.8	0±0.8	4.6	1.4±1.6



The figures in brackets denote the number of observations.
L_d is the time from the onset of the stretch to the afferent response.
L_r is the time from the completed stretch to the afferent response.

TABLE III Responses to slow stretches (initial elongation 20—25 per cent)

rise time msec	rate of deformation mm/sec	T* msec	Number of spindles ( ) number of observations	latency msec	S.D msec	deformation at the onset of the afferent action potential μ
20	0.4—0.5	0.05	12 (55)	20.0±0.8	5	9
25	0.32—0.35	0.04	5 (37)	24.0±1.4	10	8
40	0.20—0.23	0.025	6 (48)	32.0±2.0	14	7
60	0.13—0.17	0.016	8 (54)	42.0±2.5	18	6

*T threshold deformation to stretches with rise times of 1—20 msec.

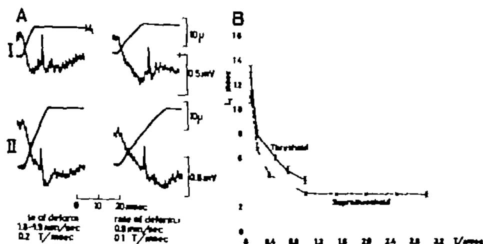


Fig. 7
A Afferent responses evoked by threshold (I) and suprathreshold (II) stretches with the same rate of deformation.
The responses occurred 1—2 msec earlier if the deformation exceeded threshold.
B Latencies (L_1) plotted against rate of deformation (T/msec)

elapsing before a propagated afferent response was elicited. With stretches of 1 msec rise time the delay was about 3 msec. This corresponds to at most 1 μ by which the deformation should be reduced in the slow stretches. The excess deformation was probably still smaller since the reduced latency of stretches which extended beyond threshold was as short as 1—2 msec (Fig. 7). Hence the threshold deformation for rates of 0.016—0.05 × T msec was at least 6 μ.

Critical slope. The stretch did not evoke an afferent response unless the rate of deformation was above a critical value. The critical slope was estimated to be just above 0.01 × T msec, because at this rate of stretch the latencies were distributed randomly (5 spindles, rise time 96—100 msec, Fig. 6B).

Suprathreshold stretches

1. First response

The latency decreased with increasing deformation to a minimum of 2.5—3 msec with rise times of 1— msec as compared to 4.2±0.3 msec when the deformation was threshold (Fig. 7, 8 and 9). The latency of the first response to a suprathreshold stretch and its S.D. were shorter with an initial elongation of 20—25 per cent than at 10—15 per cent (Fig. 8B). The shortening in latency was less pronounced with a high (1.5 × T msec) than with a low rate of deformation (0.3 × T msec, Table IV, Fig. 8).

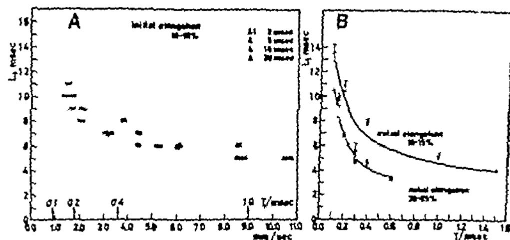


Fig 8
A The latency of afferent responses evoked by suprathreshold stretches as function of the rate of deformation at an initial elongation of 10—15 per cent. Rate expressed in threshold units per msec and in mm/sec
B Latencies (L_a) of responses to suprathreshold stretches at different initial elongations as function of the rate of deformation in threshold units (23 spindles)

TABLE IV Repetitive afferent responses to suprathreshold stretch (initial elongation 20—25 per cent)

rise time (msec)	number of spindles	minimum deformation evoking 2nd or 3rd response (threshold units)	deformation regularly evoking 2nd or 3rd response (threshold units)	interval between successive responses (msec)	latency of 1st response (msec)
		second response			
2	4	2—3	4—5	2 4	2.5—3
3	4	2 3	3—4	2 4	3—4
10	6	1—2	2—3	3—8	—8
20	6	1.5 2	2—2	9—13	9—12
		third response			
10	2	2 5	3 3.5	4 6	5—6
20	1	8	3	6 8	6—7

denotes that 30—50 per cent of the stretches evoked repetitive responses.
denotes that 80—100 per cent of the stretches evoked repetitive responses.

B Repetitive responses

Whether or not a suprathreshold stretch evoked more than *one* afferent discharge depended on the rise time and the deformation (*Fig 8B* and Table IV)

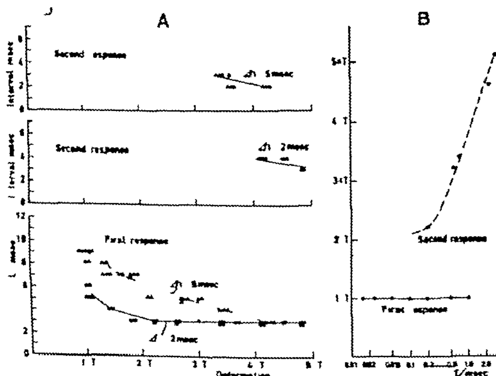


Fig. 9

A Repetitive discharges evoked by stretches with rise times of 2—5 msec.
Below. latency of the first response as function of the deformation
Above: Interval between the first and the second response at different deformations. A second response to stretches with rise times of 2 or 5 msec occurred when the deformation was more than 4 or 3 times threshold.

B The threshold of the first and the second response as function of the logarithm of the rate of stretch.

( ) Rise time of 1—5 msec Even though the deformation was more than twice threshold it did not regularly evoke a second response (Fig 9A) With increasing rate of deformation (decreasing rise time the deformation required to evoke a regular second response increased with 5 msec the deformation was 3—4 T and with 2 msec 4—5 × T (Fig. 9A and Table IV) The interval between the first and the second response was similar to the latency of the first response With further increase of deformation the interval decreased the minimum being 2 msec shorter than the shortest latency of the first response Fig 9A

) Rise time of 10—20 msec Deformations above 2 T regularly evoked two responses (Table IV) The interval between the two responses was the same as the latency of the first response and also the S D were alike Fig 10A The interval between the two responses decreased linearly as a function of the logarithm of the rate (6 spindles, Fig 10B) Most stretches above 3 T evoked three afferent responses (Table IV)

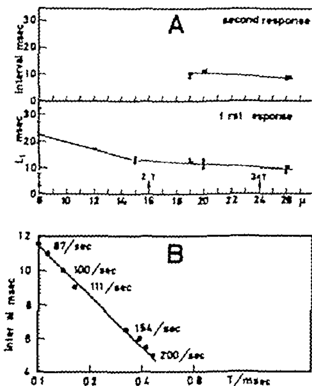


Fig. 10 Repetitive discharges evoked by stretches with rise times of 10—20 msec.
A *Below* latency of the first response to stretches with rise time of 20 msec as function of the deformation.
Above Interval between the first and the second response at different deformations. A second response occurred when the deformation exceeded 2 / T
B The intervals between the first two or three response as function of the logarithm of the rate of deformation. The figures above the points give the frequencies of the different discharges (average from 6 spindles)

The effect of spontaneous activity A spontaneous afferent discharge preceding the threshold stretch of a muscle spindle (2– 3 msec rise time) delayed or abolished the afferent response The afferent response failed to occur when the spontaneous discharge preceded a stretch by 8 10 msec a spontaneous discharge 13– 20 msec before the stretch caused the spindle to respond irregularly and with a longer latency (*Fig. 11 I and II* When spontaneous intrafusal contraction occurred 15—20 msec before a stretch the latency of the afferent response associated with the stretch was shortened (Fig 11 III Thus the intrafusal contraction acted as "internal" stretch with a deformation of 1 1.5 T Jahn 1966 and Jahn 1967 The latency of afferent responses corresponded to summation of the external and the "internal" stretches.

Discussion

Over a wide range the threshold deformation T of frog muscle spindles was independent of the rate of deformation 0.016 1.0 T msec This is consistent with the finding that the onset of the receptor potential of frog muscle spindles and the

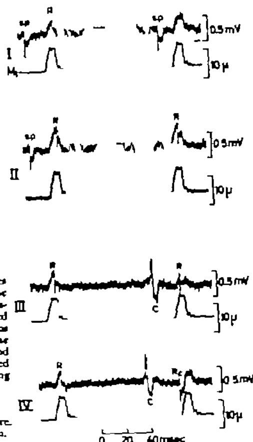


Fig. 11 Effect of spontaneous afferent discharges and spontaneous intrafusal contractions on the afferent responses to stretch. The afferent response was inhibited (I, second stretch) or delayed (II, first stretch) by preceding spontaneous afferent discharge. In III (second stretch) the latency of the afferent response was reduced and in IV (second stretch) the response was inhibited by spontaneous intrafusal contraction, preceding the stretch.

sp spontaneous afferent discharge
R afferent response
C action potential of an intrafusal muscle fibre.
R_c afferent response to intrafusal contraction.
M mechanical transient.

onset of the first propagated discharge occurred at a constant level for rates varying 100 times (Ottoson and Shepherd 1965 and Shepherd and Ottoson 1965). Also in Pacinian corpuscles (Gray and Matthews 1951) and in amphibean touch receptors (Gray and Malcolm 1951, Höglund and Lindblom 1961) the rate of deformation over a wide range was without significance for the occurrence of the first afferent response.

Denny Brown and Liddell (1927) estimated the threshold deformation of the muscle spindle in the cat's m.supraspinatus to be 8—10 μ. Lundberg and Winsbury (1959) obtained afferent responses from the cat soleus muscle when the pull applied to the tendon was less than 10 μ. These deformations are similar to the threshold measured in frog muscle spindles of m.extensor longus dig. IV stretched by 10—3 per cent (8± μ, Jahn 1965). Smaller thresholds as obtained by Ottoson (personal communication) can be expected if most of the series elastic tissue is removed such that the equatorial region is subjected to initial elongations of for example 50 per cent above equilibrium length.

The state of the intrafusal muscle bundle affects also the latency of the afferent response. When the initial elongation was increased from 10—15 per cent to 20—25 per cent the delay of responses to threshold stretches was reduced to half. Shepherd and Ottoson (1965) found a similar reduction in minimum latency when the resting length of a spindle was increased from 1 mm to 1.7 mm. The delay of the response to threshold stretches is probably an expression of the duration of the processes by which the mechanical deformation is converted into an electrical response ("mechanical filter" in muscle spindles, Houk, Cornew and Stark 1966 and mechanical "receptor delay" in tactile receptors, Catton 1966).

That the delay of the afferent response to threshold stretches was constant with rates of deformation of 0.1—1.0 × T/msec and disappeared when the deformation was slower (0.01—0.05 T/msec) agrees with findings on amphibian touch receptors (Gray and Malcolm 1951).

The shortening of the latency caused by a suprathreshold deformation (Fig. 7A and B) indicates that the excess deformation accelerates transduction as did the higher initial elongation (Fig. 8B).

The threshold in Pacinian corpuscles is about 0.5 μ (Gray and Sato 1953) similar to the threshold deformation estimated by the displacement of two graphite granula placed on the spindle capsule over the sensory region.

The thresholds determined in this study do not necessarily represent the deformations of the sensory end-bulbs because the mechanical properties of the transverse connections between the capsule and the sensory endings are unknown. Therefore it ... ore appropriate to describe the deformation of the muscle spindle in terms of ...hold units.

...mechanical deformation of the muscle spindle induces a graded receptor po...tial which in turn generates the discharge of propagated potentials (Katz 1950). Ottoson and Shepherd (1965) have analysed the regular response pattern of the isolated frog muscle spindle to linearly increasing extensions. That the rate of deformations influences the repetition of discharges agrees with their finding: at slow rates of deformation, a second and third discharge required the deformation to be 2—3 times threshold; at fast rates the deformation for the initiation of a second discharge required a much higher deformation (Fig. 9 and 10). Similar observations were made in amphibian touch receptors (Lindblom 196).

The responsiveness of the muscle spindle was affected by spontaneous intrafusal contractions (Jahn 1963 and 1966). Contractions of the muscle bundle attached to the receptor neurone facilitated also the response of stretch receptors of lobster and crayfish (Kuffler 1954). Also in amphibian touch receptors (Gray and Malcolm 1951, Höglund and Lindblom 1961) and Pacinian corpuscles (Loewenstein 1956) conditioning stretches of long duration caused threshold variations.

I wish to express my sincere gratitude to professor F. Buchthal for constant advice and encouragement, to Poul Rosenfalck, M. Sc. for valuable theoretical discussions and to Mr. ... Andersen for the design of the pulse shaping circuit and the stretch device.
The work was supported by the Muscular Dystrophy Associations of America Inc., New York and by the Danish State Research Foundation.

References

ADRIAN, E. D. and Y. ZOTTERMAN, The impulses produced by sensory nerve endings. Part 2. The response of a single end-organ. *J. Physiol.* (Lond.) 1926. *61*, 151—171.

CATTON, W. T., A comparison of the responses of frog skin receptors to mechanical and electrical stimulation. *J. Physiol.* (Lond.) 1966. *187*, 23—33.

DENNY-BROWN, D. E. and E. G. T. LIDDELL, Observations on the motor twitch and on reflex inhibition of the tendon jerk of m. supraspinatus. *J. Physiol.* (Lond.) 1927. *63*, 70—80.

GRAY, J. A. B. and P. B. C. MATTHEWS, A comparison of the adaptation of the Pacinian corpuscle with the accomodation of its own axon. *J. Physiol.* (Lond.) 1951. *114*, 454—464.

GRAY, J. A. B. and J. L. MALCOLM, The excitation of touch receptors in frog skin. *J. Physiol.* (Lond.) 1951. *115*, 1—15.

GRAY, J. A. B. and M. SATO, Properties of the receptor potential in Pacinian corpuscles. *J. Physiol.* (Lond.) 1953. *122*, 610—636.

HÖGLUND, G. and U. LINDBLOM, The discharge in single touch receptors elicited by defined mechanical stimuli. *Acta physiol. scand.* 1961. *53*, 108—119.

HOUK, J. C., CORNEW, R. W. and L. STARK, A model of adaptation in amphibian spindle receptors. *J. theoret. Biol.* 1966. *12*, 196—215.

JAHN, U., Morphological observations on living neuromuscular spindles. *Acta anat.* (Basel) 1959. *39*, 341—350.

JAHN, S. AL AHMARI, Physiological properties of isolated neuromuscular spindles. *Acta physiol. scand.* 1963. *59*, Suppl. 213, 67—68.

JAHN, S. AL AHMARI, Spontaneous intrafusal contractions and spontaneous afferent discharges as a tool in the analysis of muscle spindles. Muscular Afferents and Motor Control, ed. Granit, R. pp. 453—456. Stockholm: Almqvist and Wiksell. 1966.

JAHN, S. AL AHMARI, Spontaneous afferent discharges and spontaneous intrafusal contractions in isolated muscle spindles of the frog. *Acta physiol. scand.* 1968. *72*, 350—365.

KATZ, B., Depolarization of sensory terminals and the initiation of impulses in the muscle spindle. *J. Physiol.* (Lond.) 1950. *111*, 261—282.

KUFFLER, S. W., Mechanisms of activation and motor control of stretch receptors in lobster and crayfish. *J. Neurophysiol.* 1954. *17*, 558—574.

LINDBLOM, U., The relation between stimulus and discharge in a rapidly adapting touch receptor. *Acta physiol. scand.* 1962. *56*, 349—361.

LOEWENSTEIN, W. R., Excitation and changes in adaptation by stretch of mechanoreceptors. *J. Physiol.* (Lond.) 1956. *133*, 588—602.

LUNDBERG, A. and G. WINSBURY, Selective adequate activation of large afferents from muscle spindle and Golgi tendon organs. *Acta physiol. scand.* 1960. *49*, 155—164.

MATTHEWS, B. H. C., The response of a single end-organ. *J. Physiol.* (Lond.) 1931. *71*, 64—110.

OTTOSON, D. and G. M. SHEPHERD, Receptor potentials and impulse generation in the isolated spindle during controlled extension. *Cold Spr. Harb. Symp. quant. Biol.* 1965. *30*, 105—114.

SHEPHERD, G. M. and D. OTTOSON, Response of the isolated muscle spindle to different rates of stretching. *Cold Spr. Harb. Symp. quant. Biol.* 1965. *30*, 95—103.

Acta physiol. scand. 1968. 72. 456—468

F om the Department of Physiology University of Göteborg, Sweden

Cell Volume as a Factor Influencing Electrical and Mechanical Activity of Vascular Smooth Muscle

By

BÖRJE JOHANSSON and OLOF JONSSON

Received 26 July 1967

Abstract

JOHANSSON B. and O JONSSON. *Cell volume as a factor i fluencing electrical and mechanical activity of vascular mooth muscle* Acta physiol. scand. 1968, 72. 456—468.

The effects of ariations in the extracellular osmolarity on the electrical and mechanical activity of the isolated rat portal ei were studied. Addition of sucrose or xylose (?0—100 les/l) t the normal" solution caused nhibition of contractile acti ty which could be bed to hanges in the electrical discharge pattern. Increa ng osmolarity w th ure d ed transient inhibition, ret rn to control acti ty and, on return to normal solution, ment excitation. Reducing osmolarity by 30—60 mOsm/l by decreasing the [N] and] of the medi m aused sustained excitation with continuous spike discharge and tetanic ontraction. Comparable decreases the ionic concentrations but with osmotic substitution by sucrose or xylose had little influence on muscle acti ty Substit ting N and Cl th ure on an equiosmola basis caused excitation resembling that in hypoosmotic sol tion. If the smooth muscle cells are looked pon as osmotic lls with membranes that are freely permeable to H_2O and ure but less permeable t sugar and ions, the results ndicate consistent correla ion between cell olume and state of activ ty shrinkage associa ed th inhibition, elling w th excitation Changes in the transmembrane concentr tion gradients for K Na and Cl^- aused by the cellular olume hanges, ar not sufficient to explain the lectrical responses. Changes in ionic membrane permeability are assumed to occur

Increasing extracellular smolarit by addition of sucrose or glucose inhibit spontaneous electrical and mechanical activity in the smooth muscle of the isolated rat portal ein as demonstrated in previous report (Mellander et al 1967) E idence was presented indicating that ariations in interstitial osmolarity are of i portance in the regulation of ascular tone also in vivo It was considered of interest therefore to study in greater detail the responses of vascular smooth muscle t graded changes in the tonicity of the extracell lar fl id. The present report describes the ffects on electrical and mechanical acti it of rat portal ein produced by certain variations in the composition of the external medium which im ply osmotic strains on the smooth muscle cells.

Methods

The experiments were performed on portal vein preparations from white rats weighing 150—200 g which were killed by a blow on the neck. 5—10 mm long sections of the vein were used for mechanical experiment. The preparation was mounted in a 50 ml mantled organ bath and connected to an isometric tension transducer (Grass FTO3) for recording of contractile activity in the predominantly longitudinal musculature. The muscle was allowed to accommodate in "normal" solution (see below) under a passive tension of about 400 dyn for at least one hour before the experiment started. Simultaneous recordings of electrical and mechanical activity were done on 15 to 20 mm long sections of the portal-mesenteric vein using a sucrose-gap technique which has been described previously (Axelsson *et al.* 1967).

The normal solution as referred to below was of the following composition in mmoles/l: NaCl 122, KCl 4.73, $CaCl_2$ 2.49, $MgCl_2$ 1.19, $NaHCO_3$ 15.5, KH_2PO_4 1.19, Glucose 11.5. It was bubbled with a mixture of 96 % O_2 and 4 % CO_2 giving a pH of 7.40. The temperature was kept at 37° C.

The muscles were exposed for variable periods of time to test solutions prepared by modifying the normal solution with regard to osmolarity and ionic composition as described in the result section. In principle, hyperosmotic solutions were made by adding sucrose, xylose or urea to the normal medium, hypoosmotic solutions were obtained by reducing the amount of NaCl and, finally, modified isoosmotic solutions were made by replacing part of the NaCl of the normal medium with equimolar amounts of sucrose, xylose or urea. In calculating the osmotic substitutions and in discussing the magnitudes of the osmotic changes we consider the salts completely dissociated into free ions and the osmolar effects of non-ionic substances to be represented by their molar concentrations. The approximations involved in this are realized but they can probably be neglected in this type of study.

Results and comments

A. Portal vein responses to changes in extracellular osmolarity

1. Hyperosmotic solutions. Increasing the osmolarity of the extracellular fluid by adding sucrose to the normal solution causes inhibition of spontaneous activity in the rat portal vein as seen in the mechanical recordings of Fig. 1A and B. The first few minutes of each recording show the characteristic activity of the muscle in normal medium. Phasic contractions with a peak tension of 750 to 800 dyn appeared at regular intervals of about 20 to 25 sec. Shifting to a solution with the same ionic composition as normal solution but with osmolarity increased by the addition of sucrose (50 and 100 mmoles/l in Fig. 1A and B, respectively) reduced the frequency of the contractions in comparison with the control period. Decrease in contraction amplitude was also seen particularly in the initial phase of the 10 min exposure periods. The muscle gradually regained an activity pattern similar to that of the initial control period after return to normal solution but the early phase of recovery was characterized by relatively stronger contractions of more complex form (last part of recordings in Fig. 1A and B).

Slowing of contraction frequency was evident also at more moderate degrees of hyperosmolarity than those illustrated in Fig. 1A and B. Addition of 20 mmoles sucrose per litre to the normal solution could thus reduce frequency by 25 per cent in some experiments. The effects of the hyperosmotic solution with sucrose on the mechanical activity of the portal vein can to a great extent be explained on the basis of changes in the pattern of electrical activity as shown in our previous study (Mellander *et al.* 196

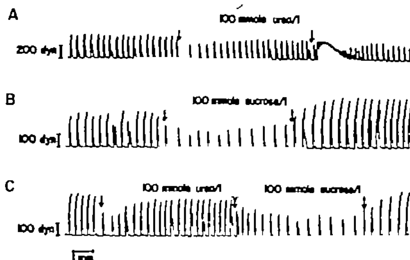


Fig. 3. Mechanical recordings from rat portal vein. Effects of hyperosmotic solutions containing sucrose or urea. Normal solution in the control periods. Note that the excitatory response on return from normal solution + urea to normal medium in A is lacking in C where normal solution + urea is replaced by normal solution + sucrose.

posure was followed by normal solution + 100 mmoles sucrose per liter. In the latter period of the record C there is a slowing of contraction frequency typical of hyperosmotic solution with sucrose but this slowing shows a relatively sluggish onset.

Hypoosmotic and modified isoosmotic solutions. Hypoosmotic solutions were prepared by reducing the concentrations of Na and Cl of the normal medium. The response of the portal vein to such a decrease in extracellular osmolarity is illustrated in Fig. 4 taken from a representative experiment. The electrical activity during the control period with normal medium (a) consisted of bursts with a duration of 5 to 8 sec at intervals of some 20 sec and each burst gave rise to a tension response of about 800 dyn.

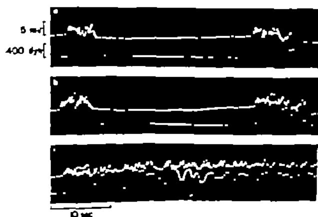


Fig. 4. Effects of reduced NaCl on portal vein. a. Normal solution with 122 mmoles NaCl/l. b. NaCl reduced to 92 mmoles/l and substituted with 60 mmoles sucrose/l. c. NaCl reduced to 92 mmoles/l without osmotic compensation.

Shifting to an isotonic solution with 92 mmoles NaCl and 60 mmoles sucrose per liter caused a transient period of slightly increased contraction frequency but within a few minutes (4b) the pattern of activity closely resembled that of the control period. Changing to a solution containing 92 mmoles NaCl/l without osmotic substitution caused depolarization, a more or less continuous spike discharge and an incomplete tetanic contraction (4c). Section b was included in this figure to demonstrate that the response occurring in c was due to the reduced tonicity and not to a specific ionic mechanism associated with the decreased $[Na^+]_o$ or $[Cl^-]_o$.

The time-course of the activity changes produced in portal vein by reduction in the NaCl of the normal solution without osmotic substitution and with osmotic substitution by different molecules is demonstrated more clearly in the mechanical experiment of Fig. 5. Record A illustrates the effects of hypoosmolarity (50 mOsm/l below normal) achieved by reducing the NaCl concentration from 122 mmoles/l to 97 mmoles/l. Record B shows the effects of the same NaCl reduction but with sucrose substitution. It is evident, as in Fig. 4, that the effects of hypoosmolarity cannot be ascribed to the accompanying reduction of the Na or Cl content of the solution. No conspicuous difference occurred when xylose in equiosmolar amounts replaced sucrose as a substitute for NaCl (5C). The exchange of 25 mmoles NaCl with 50 mmoles urea per liter (5D) resulted, however, in an excitatory response quite similar to that seen in Fig. 5A where osmolarity was re-

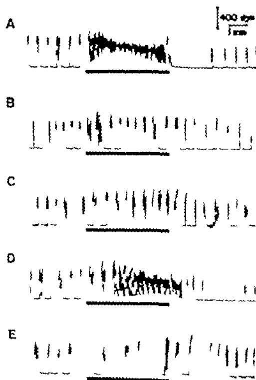


Fig. 5. Mechanical responses of rat portal vein to reductions in the NaCl of the medium without osmotic substitution and with osmotic substitution by different molecules. A, Reduction in NaCl from 122 to 97 mmoles/l without osmotic substitution. B, 25 mmoles NaCl/l of the normal solution replaced by 50 mmoles sucrose/l. C, 25 mmoles NaCl/l of the normal solution replaced by 50 mmoles xylose/l. D, 25 mmoles NaCl/l of the normal solution replaced by 50 mmoles urea/l. E, 25 mmoles NaCl/l of the normal solution replaced by 50 mmoles urea/l + 50 mmoles sucrose/l.

duced by 50 mOsm/l although the onset of the effect was slower in D The ex citation initiated by the isoosmotic solution containing 50 mmoles urea/l (D) could be prevented by adding an equivalent amount of sucrose making the solution hyperosmotic (5E)

Comments to section A

The results presented above show that quite marked changes in the spontaneous activity of the vascular smooth muscle are produced by variations in the osmotic composition of the extracellular fluid. It is also clear that the muscle can tolerate, at least for shorter periods these rather drastic changes in environment without deterioration of its electrical or mechanical activity Bozler (1962) has suggested from experiments on frog stomach that smooth muscle cells resist considerable osmotic gradients without significant changes in volume. A proper evaluation of the present results would therefore seem to require quantitative data with regard to the passage of water and solutes across the cell membranes in response to the applied osmotic forces. We have found in preliminary experiments that the portal vein loses weight in hyperosmotic solution with sucrose but the extent of the cellular volume changes has not been determined so far It seems justified to assume for the following discussion, that the vascular smooth muscle cell behaves, at least qualitatively as an osmotic cell whose membrane is freely permeable to water and urea but less permeable to xylose, sucrose and ions. The intercellular spaces in this vein preparation seem to communicate quite freely with the outer medium and the extracellular fluid can therefore, for practical purposes be looked upon as a single compartment where concentrations of solutes are equilibrated by free diffusion. Support for this latter statement is actually given by the observations in Fig. 3C and in Fig 5E in which sucrose replaces urea at the cell membranes without any marked complexities in time-course of the muscle response

The activity changes found in the present experiments show a notable and most consistent correlation to the induced changes in cell volume that may be predicted on the basis of the above assumptions Any procedure which would cause shrinkage of the cells inhibits the spontaneous activity whereas swelling causes excitatory responses. Some rather large changes in the composition of the salt solution may be made without much effect on muscle activity as long as the osmotic substitution acts to maintain constant cell volume

Sucrose when added to make the solution hyperosmotic causes a persistent shrinking of the cells and a sustained inhibition of muscle activity (Fig 1A—B) The reduction in contraction frequency and amplitude can be ascribed to changes in the pattern of electrical activity (increased burst interval and shortened burst duration) as shown in our previous report Mellander *et al* 1967 Hyperpolarization in connection with the reduced cell volume is evident under some conditions The slightly increased muscle activity seen after the hyperosmotic periods in Fig 1A and B may be due to a small osmotic swelling caused by a mechanism similar to that discussed below in connection with the urea responses of Fig. 1C and

D. Such an effect would appear if sucrose in small amounts had entered the cells during the exposure periods.

Increasing extracellular osmolarity with urea produces only a transient decrease in the volume of the cells since they will return to control size as urea, accompanied by water, enters to reach concentration equilibrium over the membranes (cf. Davson 1964). Accordingly the portal vein activity is slowed down for a brief period after urea administration and then returns to the control pattern (Fig. 1 C—D and Fig. 2 a—c). On readministration of normal solution the urea is quickly eliminated from the extracellular fluid which then becomes hypoosmotic relative to the intracellular fluid. A transient swelling of the cells occurs due to the osmotic action of the intracellular urea but, as this substance continues to diffuse out of the cells along its concentration gradient, it takes water with it and the volume returns to normal. The excitatory action of this cellular swelling is shown clearly in Fig. 1 C—D and in Fig. 2 d—e. Diffusion of urea out from the cells does not lead to an excitatory response if the transient swelling is prevented by sucrose as in Fig. 3C.

In hypoosmotic solution (reduction in NaCl without osmotic substitution) the cells swell rapidly with the entrance of water and a marked excitation develops (Fig. 4c and Fig. 5A). The excitatory response is related to the swelling and not to the ionic changes as shown by the results of NaCl substitution with non-permeant sucrose or xylose (Fig. 4b and Fig. 5B—C). Substitution of NaCl with urea does not prevent swelling since water will accompany this permeant substance as it diffuses into the cells. The degree of swelling will be comparable to that obtained without substitution (see e.g. Davson 1964). Accordingly the excitatory response in Fig. 5D is similar to that in Fig. 5A although of somewhat slower onset. This difference in onset may be due to a more rapid swelling when water enters along an osmotic gradient (A) than when its entrance depends on the inward diffusion of urea (D).

The addition of sucrose in Fig. 5E does not, of course, interfere with the uptake of urea into the cells but it cancels by its osmotic action the swelling that occurred in C. Again the activity pattern reflects the cell volume since essentially normal rhythmicity persists during the exposure period in Fig. 5E.

The changes in the mechanical activity of the portal vein which appear as a result of the osmotic forces can be ascribed essentially to changes in the pattern of electrical activity as shown in our previous study and in Fig. and 4 above. It is evident, therefore, that the mechanisms by which the cellular volume variations influence muscle activity should be sought among the factors that determine the electrical behaviour of the cell membranes. Among these factors we may consider for instance the concentration gradients of ions across the membrane, the passive ionic conductances of the membrane, the active transport of ions etc. Net influx or efflux of w nection with swelling and shrinking respectively should directly affect the cellular ion concentrations and changes in transmembrane ionic gradients may thus be the nearest explanation for the electrical responses described.

Increasing the external potassium ion concentration depolarizes the smooth muscle of the portal vein (Axelsson, Johansson and Jonsson 1966, Axelsson *et al.* 1967) indicating that the membrane potential of these vascular smooth muscle cells as of other excitable cells is greatly dependent on the potassium equilibrium potential determined by the ratio $[K^+]_i/[K^+]_e$. The possibility that cellular volume changes, produced by osmotic forces, would influence the electrical activity by altering $[K^+]_i$ was considered in a previous report (Mellander *et al.* 1967) and will be further explored in the following section of the present study.

B. Effects of extracellular potassium ion concentrations on the responses to changed osmolarity

A possible explanation for the fact that increased extracellular osmolarity inhibits the spontaneous activity of the portal vein would be that it increases the intracellular potassium ion concentration by sucking water out from the cell, thereby hyperpolarizing the cell membrane. It should be possible then to counteract the effect of increased osmolarity by increasing also the potassium ion concentration in the extracellular fluid. Solutions with increased $[K^+]_e$ were prepared by replacing NaCl with equimolar amounts of KCl.

Fig. 6 illustrates such an experiment where both electrical and mechanical activity were recorded. The addition of sucrose, 100 mmoles/l, to the normal solution (6b) resulted, as earlier described, in a decrease in the frequency and duration of

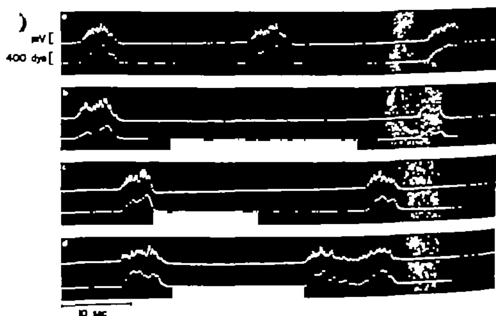


Fig. 6. Attempts to cancel the inhibitory response of the portal vein to hyperosmotic solution with sucrose by increasing $[K^+]_e$. a. Normal solution (5.9 meq K^+/l). b. Normal solution + 100 mmoles sucrose/l. c. Solution with 11.8 meq K^+/l + 100 mmoles sucrose/l. d. Solution with 17.7 meq K^+/l + 100 mmoles sucrose/l.

5 mV[
400 dyn[

10 sec

Fig. 7 Attempts to cancel the inhibitory response of the portal em to hyperosmotic solutions th sucrose by increasing [K]$_o$ at high level of external potassium. a. Solution with 24 meq K/l. b. Solution th 24 meq K/l + 100 mmoles sucrose/l. Solution with 30 meq K/l + 100 mmoles sucrose/l. d. Solution with 36 meq K/l + 100 mmoles sucrose/l.

the bursts and in the contraction amplitude as compared to control in normal medium (6a) When the potassium ion concentration of the solution was increased by 100 per cent (that is from 5.9 to 11.8 meq/l) at the same time as sucrose was added, the inhibition of the activity became less conspicuous (6c). There was still a decrease in the frequency of the bursts as compared to control but th contraction amplitude was roughly normal Record 6d illustrates the activity after 5 minutes in a solution containing 17 meq K/l, 3 times the normal concentration, and 100 mmoles sucrose/l. Bursts of longer duration ppeared in this solution and the intervals between the bursts were comparable to those of the control period Experiments like that of Fig. 6 have shown c nsistently that at the normal level of external potassium, it is necessary to increase [K] by at least 100 per cent in order to cancel the influence of a 30 per cent increase smolarity

In the experiment illustrated in Fig 7 the pr tal ein was upert sed w th sol tion containing four times the normal potassium ion oncentration bout 24 meq K/l. The burst duration nd ontraction amplit de wer c nsiderably increased n this solution (7a) Th additio of 100 mmoles sucrose pe liter 7b decreased the burst duration and ncre sed the in ervals between the bursts When the potassium ion concentration was increased simultaneousl w th th smolarit from 4 to 5 times the normal [K] 7 partial recovery towards the pattern of Fig occurred. It thus did not seem t be q ite sufficient t ... e the actual potassium ion concentration by 25 per cent to outbalanc th ffect of 10 mmoles sucrose However in Fig 7d, where the external potassium oncent tion w in creased by 50 per cent (from about 24 to about 35 meq l t the same ...

Increasing the external potassium ion concentration depolarizes the smooth muscle of the portal vein (Axelsson, Johansson and Jonsson 1966, Axelsson *et al.* 1967) indicating that the membrane potential of these vascular smooth muscle cells as of other excitable cells is greatly dependent on the potassium equilibrium potential determined by the ratio $[K^+]_i/[K^+]_o$. The possibility that cellular volume changes, produced by osmotic forces, would influence the electrical activity by altering $[K^+]_i$ was considered in a previous report (Mellander *et al.* 1967) and will be further explored in the following section of the present study.

B. Effects of extracellular potassium ion concentrations on the responses to changed osmolarity

A possible explanation for the fact that increased extracellular osmolarity inhibits the spontaneous activity of the portal vein would be that it increases the intracellular potassium ion concentration by sucking water out from the cell, thereby hyperpolarizing the cell membrane. It should be possible then to counteract the effect of increased osmolarity by increasing also the potassium ion concentration in the extracellular fluid. Solutions with increased $[K^+]$ were prepared by replacing NaCl with equimolar amounts of KCl.

Fig. 6 illustrates such an experiment where both electrical and mechanical activity were recorded. The addition of sucrose, 100 mmoles/l, to the normal solution (6b) resulted, as earlier described, in a decrease in the frequency and duration of

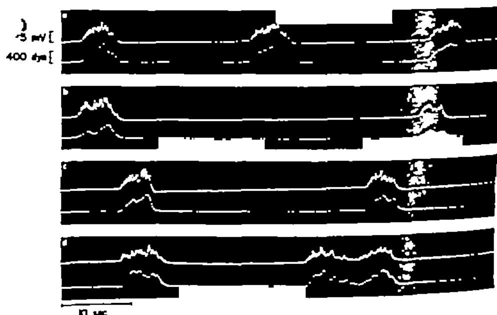


Fig. 6. Attempts to cancel the inhibitory response of the portal vein to hyperosmotic solutions with sucrose by increasing $[K^+]_o$. a. Normal solution: 5.9 meq K/l. b. Normal solution with 100 mmoles sucrose/l. c. Solution with 11.8 meq K/l + 100 mmoles sucrose/l. d. Solution with 17.7 meq K/l + 100 mmoles sucrose/l.

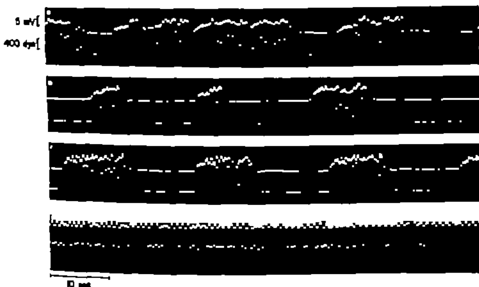


FIG. 7. Attempts to cancel the inhibitory response of the portal vein to hyperosmotic solutions with sucrose by increasing $[K^+]_o$ at a high level of external potassium. a. Solution with 24 meq K^+/l. b. Solution with 24 meq K^+/l + 100 mmoles sucrose/l. c. Solution with 30 meq K^+/l + 100 mmoles sucrose/l. d. Solution with 36 meq K^+/l + 100 mmoles sucrose/l.

the bursts and in the contraction amplitude as compared to control in normal medium (6a). When the potassium ion concentration of the solution was increased by 100 per cent (that is from 5.9 to 11.8 meq/l) at the same time as sucrose was added, the inhibition of the activity became less conspicuous (6c). There was still a decrease in the frequency of the bursts as compared to control but the contraction amplitude was roughly "normal". Record 6d illustrates the activity after 5 minutes in a solution containing 17.7 meq K^+/l, 3 times the normal concentration, and 100 mmoles sucrose/l. Bursts of longer duration appeared in this solution and the intervals between the bursts were comparable to those of the control period. Experiments like that of Fig. 6 have shown consistently that at the normal level of external potassium, it is necessary to increase $[K^+]$ by at least 100 per cent in order to cancel the influence of a 30 per cent increase in osmolarity.

In the experiment illustrated in Fig. 7 the portal vein was superfused with a solution containing four times the normal potassium ion concentration, about 24 meq K^+/l. The burst duration and contraction amplitude were considerably increased in this solution (7a). The addition of 100 mmoles sucrose per liter (7b) decreased the burst duration and increased the intervals between the bursts. When the potassium ion concentration was increased simultaneously with the osmolarity from 4 to 5 times the normal $[K^+]$ (7c), partial recovery towards the pattern of Fig. 7a occurred. It thus did not seem to be quite sufficient to increase the actual potassium ion concentration by 25 per cent to outbalance the effects of 100 mmoles sucrose. However, in Fig. 7d, where the external potassium concentration was increased by 50 per cent (from about 24 to about 35 meq/l) at the same time as



Fig. 1 Cat 2.9 kg. The distribution of tipyrine-C m th small intestin fi sec after an intra-arterial injection of the tracer as revealed by toradiography (middle panel) The corresponding histological section and densitometric tracing are illustrated in the upper nd lower panels, respectively Thin, horizontal line in lower panel indicates densitometric base line. Recorded total intestinal blood flow amounted to 90 ml/min × 100 g. S = submucosa. C = rypts V = villi.

bution of the other isotope used in the same experiment (cf Quimby and Feitelberg 1963 p. 269) The time of ounting was prolonged so that the coefficient of ariation amounted to 3—3 per cen det rmined by the equation given by Quimby and Feitelberg (1963) No orrection for the self absorption was made since the tissue samples in the present experiments small

Results

.uto ad g aphic findings

The middle panel of Fig 1 illustrates a representativ autoradiograph of mucosa and submucosa of an intestinal segment removed approximately five sec a an i.a. injection of antipyrine-C The corresponding histological section : densitometric tracing are shown in the upper and lower panels, respectively In experiment the total intestinal blood flow was 90 ml min × 100 g It is evid from the figure that the greatest concentration of antipyrine-C was localized to tissue between the crypt of the mucosa It is also noted that a declining concent tion of the tracer existed towards the tips of the villi, the concentration in the t being abo 1/3 1/4 that of the crypt Furthermore Fig 1 clearly demonstra that the densitometric tracing provides an accurate and detailed picture of t relative concentration of the tracer in the t ssue Hence the autoradiographs a their corresponding histological sections will not be ncluded in the follow figures

The comparativ ly low concentration of ntipyrine-C in the illi, noted at l blood flow level Fig 1 and upper left panel of Fig 2 might reflect an abse l blood flow in the villi at the esting flow level The observation illustrated i the upper middle panel of Fig makes this explanation less plausible It is also in that panel that the amount of blood labelled with India ink wa of ant— s mewhat larger in th t ps f the illi than in the rest of the mucosa at low l level

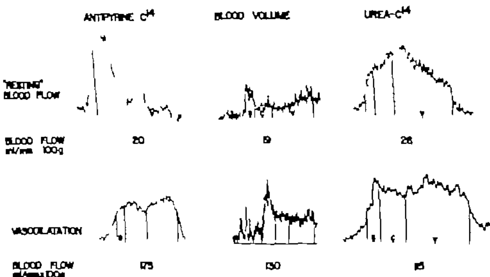


Fig. 2. Densitometric tracings of autoradiographs indicating the distribution of antipyrine-C (right section) and urea-C^{14} (left section) in the small intestine fiv sec after i.a. administration of the tracers. The distribution of the vascular olume as indicated by India ink is illustrated in he middle section. The experiments were perfomed during resting conditions and during vasodilatation induced by i.a. infusions of isopropylnoradrenaline. The recorded total intestinal blood flows are indicated in the figure. Thin, horizontal lines indicate densitometric baselines. S = submucosa C = crypts V = villi.

The distribution of a lipid insoluble substance, urea-C is shown in the upper right panel of Fig 2. It can be seen that the concentration of urea-C in the tips of the villi was about half of that in the bases of the villi.

Th pattern of distribution of antipyrine-C and urea-C in the intestinal mucosa and submucosa, noted at resting" blood flows, differed from that observed during vasodilation induced by a constant, a. infusion of isopropylnor adrenaline. This is illustrated in the lower panels of Fig 2 which show the distribution of antipyrine-C and urea-C in the inner layers of the intestine approximately fiv sec after an i.a. injection of the tracers when venous outflow of blood amounted to 175 (antipyrine-C left panel) and 115 ml min $\times$ 100 g (urea-C right panel) respectively. It is noted that the concentration of the two tracers was fairly constant throughout the mucosa and submucosa during asodilatation. The relativ distribution of blood olume within the submucosa and mucosa during asodilatation is also illustrated in the lower middle panel of Fig 2.

Fig 3 illust tes the distribution in the intestinal mucosa and submucosa of antipyrine-C (left section) and urea-C (right section) 8—13 min after an i.a. injection of the tracers. The experiments of the upper panels were performed during resting intestinal blood flow and those of the lower panels during vasodilatation induced by infu ion f isopropylnoradrenaline. The total intestinal blood flows

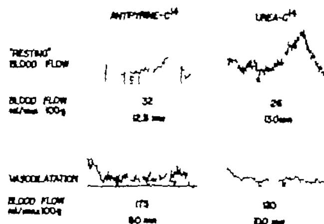


Fig. 3. Densitometric tracings of autoradiographs indicating the distribution of antipyrine-C14 (right section) and urea-C14 (left section) in the small intestine 8.0—13.0 min after i.a. administration of the tracers. The experiments were performed during "resting" conditions and during vasodilatation induced by i.a. infusion of isopropyl-noradrenaline. The recorded total intestinal blood flow and the times of removal are indicated in the figure. Thin, horizontal lines indicate densitometric baselines. S = submucosa, C = crypts V = villi.

recorded are indicated in the figure. It is shown that the distribution pattern was similar for the two tracers at corresponding blood flow levels. A distinct difference was, however, noted between the densitometric tracings of the segments removed at "resting" blood flow as compared to those extirpated during vasodilatation. Thus, a peak concentration of the tracers in the tips of the villi and continuous decline towards the bases of the villi were observed at "rest". During vasodilatation quite another distribution pattern was present, the concentration of the two solutes being highly constant throughout the mucosa and submucosa.

By comparing the appearance of the autoradiograph of the intestinal segment, removed about five sec after an i.a. injection of antipyrine-C14 with that of the segment taken out 1—2 min after the injection, it was possible to localize from what part of the mucosa and the submucosa the most rapid wash-out of the tracer occurred. Such an analysis was performed at different outflow levels as illustrated in Fig. 4. It can be seen that elimination of antipyrine-C14 appears to be fastest from the tissue between the crypts at the flow level of section A (20 ml/min × 100 g) and from the bases of the villi and to some extent also from the adjacent tissue between the crypts at the outflow level of section B (60 ml/min × 100 g). In section C the venous outflow amounted to about 100 ml/min × 100 g and the wash-out of the tracer was very fast from the tips of the villi and from a region located in the submucosa and in adjacent parts of the mucosa. In the experiment of section D of Fig. 4 performed at a venous outflow of 175 ml/min × 100 g a rapid wash-out of antipyrine was noted from parts of the submucosa and adjacent parts of the mucosa (cf. section C).

Results were presented in recent papers by Kampp, Lundgren and Sjöstrand (1967) and Kampp and Lundgren (1967) which seemed to indicate that the rapid elimination of i.a. injected antipyrine-C14 (and krypton) from the submucosa and adjacent parts of the mucosa noted during vasodilatation, reflected blood flow through an extremely well perfused portion of the intestine. It seems, therefore reasonable to assume that the rapid elimination from corresponding parts of the

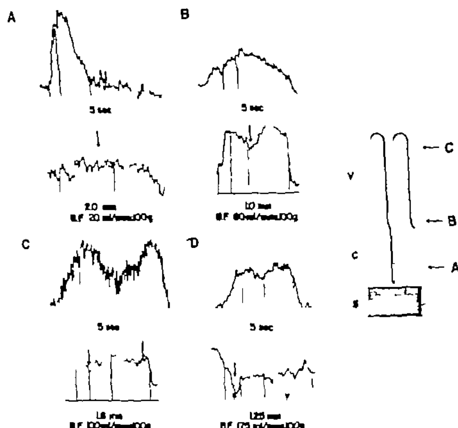


Fig. 4. Densitometric tracings of autoradiographs indicating the distribution of antipyrine-C[14] in the small intestine fr sec and 1—2 min after i.a. injection of the tracer performed at arious levels of total intestinal blood flow (B F) Thin, horizontal lines indicate densitometric baselines. Arrows indicate the localization of the most rapid wash-out of the tracer A schematic drawing of the intestinal mucosa and submucosa is shown in the right part of the figure. S = submucosa, C = crypts V = illi For details, see text

intestinal wall observed during asodilatation in the present tudy (sections C and D of Fig 4 dotted arrows) mirrors the same phenomenon

It was, however also noted by these authors that a considerable amount of i.a. injected krypton[85] was eliminated ery rapidly from the intestine even at resting' total intestinal blood flow To judge from the results of the present study per formed with antipyrine-C the localization of this wash-out varied with the magnitude f blood flow It seemed to mov from the tissue between the crypts in section A (total intestinal blood flow 20 ml min × 100 g) to the tips of the illi in section C (total intestinal blood flow 100 ml min × 100 g) This finding is also illustrated in the schematic histological picture included in Fig 4

Experiments similar to those described in connection with Fig. 4 utilizing instead urea-C^{14} were also performed at venous outflows ranging from 28 ml/min × 100 g to about 130 ml/min × 100 g. Any findings corresponding to those illustrated in Fig. 4 were then not discernible. This may possibly be explained by a comparatively small countercurrent diffusion exchange of urea-C^{14}.

B. The distribution of 4-iodoantipyrine and rubidium in the intestinal villi

The distribution in the intestinal villi of a lipid soluble (4-iodoantipyrine labelled with I^{125} or I^{131}) and a lipid insoluble (Rb^{86}) substance was also studied at "resting" blood flow by determining the ratio between the amounts of the two solutes in three or four transverse sections of the villi by means of a well-type scintillation detector. The results are summarized in Fig. 6 and 7 in which the ratio at the base has arbitrarily been set at 1.00. In the experiments, illustrated in Fig. 5, the infusions of a solution containing 4-iodoantipyrine I^{131} and Rb^{86} were made via a catheter in the left femoral vein. The villi were here cut into three transverse sections. In order to secure a higher counting rate and, hence, a smaller coefficient of variation of the number of counts within a reasonable time of counting, a second series of experiments was performed in which a mixture of 4-iodoantipyrine I^{125} and Rb^{86} was infused via a catheter in the superior mesenteric artery. In the latter experiments the villi were cut in four transverse sections (Fig. 6).

It is evident from the two figures that the ratio between the lipid soluble and lipid insoluble substances showed a declining trend from the bases towards the tips of the villi. The ratio of the two tip sections of both figures is significantly different than 1.0. In control experiments, in which the intestinal segments were allowed to

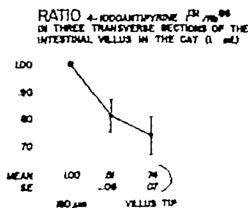


Fig. 5

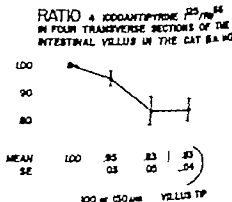


Fig. 6

Fig. 5. The diagram is based on [illegible] experimental runs performed at "resting" blood flow levels. The ratio at the base of the villi was arbitrarily set to 1.00. Dots indicate mean values, bars standard error of the mean. The [illegible] values of the two tip sections deviate significantly from 1.00 (p < 0.05 and p < 0.01), respectively.

Fig. 6. The diagram is based on [illegible] experimental runs performed at "resting" blood flow levels. The ratio at the base of the villi was arbitrarily set to 1.00. Dots indicate mean values, bars standard error of the mean. The [illegible] values of the two tip sections deviate significantly from 1.00 (p < 0.010).

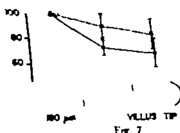


Fig 7

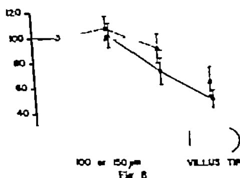


Fig 8

Fig. 7 *Relative amount (i.e. relative concentration)* deduced from the data of Fig 5 (for details, see text) The relative amount at the base of the villus was arbitrarily set to 100 Dots indicate mean values, bars standard error of the means. The average values for antipyrine of the two tip sections deviate significantly from 100 ($p = 0.001$ and $p < 0.025$ respectively) The mean values for rubidium of the sections do not deviate significantly from 100.

Fig. 8. Relative amount (i.e. relative concentration) deduced from the data of Fig. 6 (for details, see text) The relative amount at the base of the villus was arbitrarily set to 100 Dots indicate mean values, bars standard error of the means. The average values for antipyrine of the two tip sections deviate significantly from 100 ($p < 0.05$ and $p < 0.001$ respectively) The mean value for rubidium of the tip section deviates significantly from 100 ($p < 0.025$)

thaw and kept at 20—50 C for half an hour it was shown that no such declining ratio was longer present.

Since the findings illustrated in Fig. 5 and 6 may be explained in several ways they call for some comments here. The results may for example, mainly reflect a decreasing concentration of 4-iodoantipyrine from the bases towards the tip of the villi Or it may predominantly mirror an increasing concentration of rubidium from the tips to the bases. In an attempt to analyse in detail the distribution of the two substances in the villi, the data were treated in the following way Provided the counting efficiency was similar the counting rate of the different transverse sections was a relative measure of the amount of tracer present in the tissue sections. If it is assumed that the volume of the transverse sections cut by the freezing microtome was approximately equal in one and the same experiment the relative mean concentration of the tracer in the tissue section was also reflected in the counting rate. It seems therefore possible to estimate the differences in concentration of the two tracers in the different transverse sections of the villi by comparing counting rates.

Such an analysis was performed on the two experimental series of Fig 5 and 6 and the results are illustrated in Fig 7 and 8, in which the relative amount (i.e. the relative concentration) has arbitrarily been set to 100 at the bases of the villi. It is evident from both figures that the concentration of 4-iodoantipyrine, as well as rubidium showed declining trend from the bases towards the tips of the v

The decrease in concentration seems, however to more pronounced for 4-iodo-antipyrine than for rubidium. In fact the relative amount of rubidium deviates significantly from 100 only in the tip section of Fig 8, while the amount of antipyrine of the two tip sections in both figures is significantly different than 100.

Discussion

The results of the present study indicate that intravascularly administered antipyrine (labelled with C^{14} I^{125} and I^{131}) and to some extent also urea-C^{14} and rubidium^{86} were excluded from the villi at "resting" flow (Fig 1 2, 4—8) although the villi obviously were perfused with blood (Fig 2) In addition, it was demonstrated that antipyrine-C^{14} and urea-C^{14} were comparatively slowly eliminated from the villi during resting intestinal blood flow to judge from the relatively high concentration of the two tracers remaining in the tips of the villi 12—13 min after the injection (Fig 3) When the intestinal vascular bed was dilated by a constant, i.a. infusion of isopropylnoradrenaline, the concentration of antipyrine-C^{14} and urea-C^{14} seemed to be more or less constant throughout the mucosa immediately after the injection as well as 8—10 min later (Fig 5) The above described findings are all in accordance with the countercurrent hypothesis, as discussed in the introduction.

Fig 4 offers further evidence for the countercurrent exchange of antipyrine-C^{14} It is shown in this figure that the localization of the fastest elimination rates of this tracer varied with the magnitude of blood flow At low venous outflow it was localized to the tissue between the crypts and it seemed to move towards the tips of villi as flow was augmented to about 100 ml min × 100 g According to the countercurrent hypothesis this observation is explained in the following way It seems reasonable to assume that mean transit time of blood through the vasculature of the mucosa was shortened as total intestinal blood flow increased Although the diffusion of the tracer between the two limbs of the mucosal vascular loop is very fast, it certainly takes a definite length of time As the linear rate of mucosal blood flow was increased, the main part of the countercurrent diffusion of antipyrine-C^{14} took place more and more distal in the vascular tree i.e. it moved from the tissue between the crypts towards the tips of the villi.

The capillary permeability of substances is largely dependent on two factors, i.e. the dimension of the molecule and the lipid solubility of the substance (cf Landis and Pappenheimer 1963) Table I gives molecular weights (indicating the molecular dimensions) and the oil water partition coefficient (indicating the degree of lipid solubility) of the substances used in the present study The corresponding data on oxygen and krypton are also included for comparison It was observed in the present study that antipyrine-C and 4-iodoantipyrine were more efficiently excluded from the tips of the villi than urea-C and rubidium at resting total intestinal blood flow In view of the data presented in the table it seems reasonable to ascribe this finding to differences in lipid solubility (oil water partition coefficient) It should, however be pointed out that regional differences in tissue concentrations of urea

TABLE I.

	Mol.Wt.	Oil/Water partition coefficient		Reference
Rubidium	86	—		
Urea	60	0.00015	(20 C)	Renkin 1952
Antipyrine	188	0.07—0.09	(37 C)	Renkin 1952
4-iodoantipyrine	313 / 319	0.07—0.09	(37 C)	
Oxygen	16	5.0	(37 C)	Lawrence et al. 1946
Krypton	85	9.6	(37 C)	Lawrence et al. 1946

The oil/water partition coefficient of antipyrine and 4-iodoantipyrine has been assumed to be identical.

and rubidium might be due to regional differences in linear rate of flow since there are reasons to believe that the extraction of lipid insoluble substances from blood is dependent on the velocity of blood flow. Such an incomplete extraction of the solute may also imply that a comparatively high concentration of the tracer is present in the veins immediately after the intra arterial injection, a possibility which further complicates the analyses of the autoradiographs.

The data on oxygen and krypton presented in Table I show that the oil/water partition coefficients of these two solutes are about one hundred times larger than that of antipyrine. It seems, therefore, reasonable to assume that oxygen and krypton are shunted in the countercurrent exchanger of the intestinal mucosa to a greater extent than antipyrine.

Results qualitatively similar to those of the present study have been reported in investigations on the distribution of i.a. administered substances in the medulla of the kidney which has been extensively studied (for a recent review of the literature, see Lever 1965). Thus, potassium, rubidium, krypton and oxygen have been shown to be largely excluded from the papilla in all probability because of the counter current exchange that takes place between the ascending and descending limbs of the vasa recta.

The physiological implications of the present findings have not, as yet, been fully clarified. It seems, however probable that the proposed countercurrent mechanism forms an automatic "damping" device to hinder too rapid intestinal absorption. Results presented in a recent paper by Kampp, Lundgren and Sjöstrand (1967) indicate, in accordance with this, that krypton and antipyrine are slowly eliminated from the intestinal lumen at "resting" blood flow levels. Such a mechanism may thus, prevent sudden and large variations in the osmolarity of the blood leaving the small intestine, protecting the liver and the rest of the organism from osmotic shocks.

Easily diffusible substances, which reach the intestinal mucosa via the arterial—

blood, may to a large extent be shunted extravascularly from the arterial to the venous limbs of the vascular loops of the mucosa in the presence of an arterio-venous concentration difference This mechanism prevents the substance from diffusing out into the intestinal lumen. Such an extravascular short-circuiting may be of particular importance for the oxygen supply of the liver (*cf* Kampp Lundgren and Nilsson 1967) It should be pointed out in this connection that the permeability characteristics of the capillary wall are, generally speaking, similar to that of the intestinal epithelium although the size of the pores of the intestinal epithelium (4—8 Å *cf* Fordtran and Dietschy 1966) is smaller than that of the capillary wall (35—40 Å in skeletal muscle Landis and Pappenheimer 1963) Those substances which are most easily exchangeable across the epithelial lining of the intestinal lumen (lipid soluble substances of small molecular weight) will therefore also be most significantly affected by the countercurrent exchange mechanism.

This work was supported by grants from the Swedish Medical Research Council (B67 14X-16-03B) from Air Force School of Aerospace Medicine through the European Office of Aerospace Research (OAR) United States Air Force under Contract AF 61(052) 752, from U.S. Public Health Service (HE-05675-06) and from the Faculty of Medicine, University of Goteborg.

References

FORDTRAN J S and J M DIETSCHY Water and electrolyte movement in the intestine. *Gastroenterology* 1966 *50* 263—285

HELLER, A Über die Blutgefässe des Dünndarmes. *Ber sächs Ges* 1872 *24* 165—171

KAMPP M and O LUNDGREN E idenc for countercurrent exchange in intestinal illi *Acta physiol sc nd* 1966 *68* Suppl. 277 103

KAMPP M O LUNDGREN and N J NILSSON Extravascula short-circuiting of oxygen indicating countercurrent exchange in the intestinal illi of the cat. *Experientia* (Basel) 1967 *23* 197—198

KAMPP M O LUNDGREN and J SJOSTRAND On the components of the Kr^85 wash-out curves from the small testine of the cat *Act phy iol scand* 1968 *72* 257—281

LANDIS, E M and J R PAPPENHEIMER. Exchange of substances through the capillary walls I *Handbook of Physiol* 1963 Section (Circulation) Vol II 961—1034

LAWRENCE, J H W F LOOMIS C A TOBIAS and F H TURPIN Preliminary observations on the narcotic ffect of Xenon with eview of values for solubilities of gases in water and oils. *J Phy iol* (Lond) 1946 *105* 197—204

LAVEN, A F The asa recta nd countercurre t multiplication *Act med scand* 1965 *178* Suppl. 434 1—43

NIBOKA, T 1927 C ted by Spanner 1932

QUIM E H and S FEITELBERG *Radioactiv sot pe in m dicin d biology* Lea and Febiger Philadelphia 1963

RENKIN E M Capillary permeability to lipid soluble molecules *Amer J Physiol* 195—168 538—545

SPANNER, R. Neue Bef nde be die Blutwege der Darmwand und ihre funktionelle Bedeutung *Morph Jb* 193 69 394—434

THURAU K and P DEETJEN Die Diurese bei art iellen Druc ksteigerungen. *Pflügers A h f Phy iol.* 1962 4 56—580

Acta physiol. scand. 1968. 72. 481—491

From the Institute of Medical Physiology A, University of Copenhagen, Denmark

Glucose Metabolism and Response to Insulin of Isolated Fat Cells and Epididymal Fat Pads

By

JØRGEN GLIEMANN

Received 21 August 1967

Abstract

GLIEMANN, J. *Glucose metabolism and response to insulin of isolated fat cells and epididymal fat pads.* Acta physiol. scand. 1968. 72. 481—491.

The glucose uptake and the conversion of glucose-1-^{14}C to ^{14}C-carbon dioxide and ^{14}C-triglyceride by epididymal fat pads and fat cells isolated after treatment with collagenase was studied with varying concentrations of glucose in the incubation medium. The isolated fat cells took up and metabolized slightly more glucose than the fat pads at low concentration (0.1 mg/ml) but several fold more than the fat pads at high concentration (1.6 mg/ml). In the presence of insulin, the isolated cells took up and metabolized 2—3 times more glucose than the fat pads at the low medium glucose concentration and about the same as the fat pads at the high medium glucose concentration. The isolated cells were about 10 times more sensitive to insulin than the fat pads. The isolated cells responded to a concentration of 1.25 μU of insulin per ml and maximal effect was obtained with a concentration of 20 μU per ml. The equivalent figures for the epididymal fat pads were about 10 μU per ml and 320 μU per ml. Isolated fat cells, preincubated with insulin (100 μU/ml) and then washed, were stimulated for 15—30 min whereas epididymal fat pads, similarly preincubated with insulin (1 000 μU/ml) and washed, remained fully stimulated throughout a 2 hr incubation period. The differences in glucose metabolism of isolated fat cells and epididymal fat pads could be partially explained by a limitation of the glucose diffusion into the fat pad. In addition, the isolated cells seemed more permeable to glucose than fat cells of the "intact" tissue.

Most of the knowledge on the metabolism of the adipose cell, which has accumulated during the past decades, has been derived from studies using rat epididymal fat pads. This tissue is very sensitive to insulin and various effects of the hormone have been studied. The resulting data have cast some light on the physiological importance of adipose tissue and have also provided an important basis for various theories for the mode of action of insulin.

Isolated fat cells were first prepared and studied by Rodbell (1964). It was found that free fat cells metabolized glucose in a pattern similar to that of the "intact" adipose tissue both in the absence and in the presence of insulin.

In this paper some marked quantitative differences are reported between the insulin effect on the glucose metabolism of free fat cells and of epididymal adipose

tissue. The study of these differences was undertaken in an attempt to describe the effects of insulin on the isolated fat cell preparation which seems attractive as a tool for the study of the mechanism of action of insulin.

Material

Male Wistar rats were fed ad libitum and weighed 110—130 g when used, unless otherwise stated. Disposable plastic vials, used for incubation and for scintillation counting, were obtained from B.N. Plastics, Herlev, Denmark. Glucose-1-^{14}C, specific activity 16.5 μC per mg, was purchased from The Radiochemical Center, Amersham, England. Collagenase, lot no. 858—0290, was supplied by the Sigma Company. Human serum albumin (Swiss Red Cross) was prepared as previously described (Gliemann 1967). Beef insulin (10 times crystallised, lot no. 018864, activity 24.4 I.U. per mg) and guinea pig antibeef insulin serum were gifts from The NOVO Research Institute.

Methods

The preparation and incubation of isolated fat cells was performed as described (Gliemann 1967a). For the tissue incubations, the fat pads were divided in halves and distributed with 2 proximal parts and 2 distal parts in each group of 4 basal incubations. The paired tissue segments were incubated in the corresponding insulin containing flasks. The adipose tissue slices were prepared by cutting the epididymal fat pads into slices of 0.3 mm thickness with a McIlwain-Buddle tissue chopper. The fat pads and the tissue slices were incubated under conditions identical to those of the fat cell incubations. Each incubation vial contained 0.1 μ-Curie of glucose-1-^{14}C.

The glucose uptake was calculated from the disappearance of glucose from the medium. After the incubation, the medium was carefully mixed with the condensed water located to the sides and the neck of the incubation flask. The cells were allowed to float and 500 μl of medium underneath the cell layer was removed with a needle through the stopper. This aliquot was used for determination of the glucose content with the glucose oxidase method (Bergmeyer and Bernt 1963).

^{14}C-carbon dioxide was collected as described (Gliemann 1967a). Control experiments showed that 15.8 per cent (S.D. 1.1, n = 5) of the ^{14}C-carbon dioxide was present in 500 μl of the buffer when the incubations were carried out in 2 ml of medium. Accordingly, the ^{14}C-carbon dioxide production was corrected in the experiments where glucose uptake was measured.

The triglycerides were extracted from 1 ml of cell suspension by the addition of 5 ml of 2-propanol-heptane 4:1. The tissues were homogenized in the same extraction mixture. The mixture was split into 2 phases by addition of heptane and water (Dole and Meinertz 1960). The upper phase was washed twice with 5 ml of water and 1—2 ml was either dried on a planchet (for weighing) or added to 18 ml of scintillation fluid. No quenching was detected in the subsequent liquid scintillation counting. The radioactivity was assayed in a Packard Tri-Carb liquid scintillation counter. The scintillation fluid was toluene with 2,5-diphenyloxazole (5 g/l) and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene (0.3 g/l). All samples were measured until at least 5 000 counts had accumulated.

Results

Uptake of glucose and conversion of glucose-1-^{14}C to ^{14}C-carbon dioxide and ^{14}C-triglyceride by free fat cells and epididymal fat pads

About 60—100 per cent of the glucose uptake was accounted for as carbon dioxide production and synthesis of triglyceride both in the free cells and in the tissue and both at a low (0.1 mg/ml) and a high (1.6 mg/ml) concentration of glucose in the medium. At the low glucose concentration, the free cells metabolized slightly more glucose per mg triglyceride than the tissue in the absence of insulin and 2—3 times

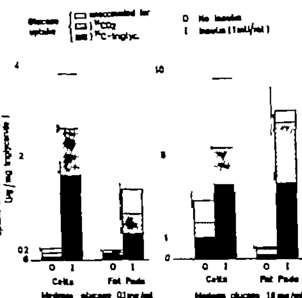


Fig. 1 The effect of glucose and insulin on the uptake and conversion of glucose-1-^{14}C to ^{14}C-carbon dioxide and ^{14}C-triglyceride by isolated fat cells and epididymal fat pads.

Isolated fat cells were prepared from 10 fat pads and 14 pads were divided in halves and quarters. The cell incubations contained 2 ml of medium and 39.1 ±4 mg of triglyceride per sample in the absence of insulin (basal incubation). When insulin was present, the cell suspension was diluted 1:10 (medium glucose 0.1 mg/ml) or 1:2 (1.6 mg/ml). The tissue incubations contained 1 quarter fat pad (medium glucose 0.1 mg/ml, plus insulin), 1 half pad (0.1 mg/ml, no insulin and 1.6 mg/ml, plus insulin) and 4 half fat pads (1.6 mg/ml, no insulin). The fat pads contained 121—163 mg of triglyceride. The results are the mean of 4 incubations ± S.D. Note the difference in scales.

more than the tissue in the presence of insulin. Therefore, the effect of insulin (per cent increase above basal level) was higher on the cells than on the intact tissue. On the other hand, when a high concentration of glucose (1.6 mg/ml) was present in the medium, the free cells metabolized about 5 times more glucose than the tissue in the absence of insulin but about the same in the presence of insulin. Hence the effect of insulin was lower on the cells than on the tissue (Fig. 1).

For technical reasons, the glucose uptake could be measured only in a relatively narrow range of glucose concentrations. The conversion of C-1 labeled glucose to carbon dioxide and triglyceride by isolated fat cells and epididymal fat tissue was compared over a wider range (Table I). With increasing concentrations of glucose in the medium, the basal carbon dioxide production and triglyceride synthesis increased more in the isolated cells than in the tissue. In the presence of insulin the glucose metabolism of the tissue increased in response to rising glucose concentrations throughout the whole range. In contrast, the metabolism of the isolated cells was only increased little when the medium glucose rose above 0.8 mg per ml.

The glucose utilization of isolated cells increased with rising extracellular concentrations of glucose and there was no tendency to reach a plateau (Fig. 2 A). The insulin-treated cells approached their maximal glucose metabolism at a concentration of 0.4 mg of glucose per ml. The insulin-induced conversion of glucose into carbon dioxide plus triglyceride increased with rising extracellular concentrations of glucose up to 0.4 mg per ml, reached a plateau, and finally at very high concentrations of glucose decreased (Fig. 2 B). The same pattern was found when glucose-U-C was used instead of glucose-1-C.

TABLE 1 The ffect of glucose nd Insulin on the metabolism of glucose 1 ¹⁴C by isola ed fat cells 12 rats were used and the cells were prepared from half of the fat pads. The paired The cell incubations contained 8.1 ± 0.6 mg of triglyceride per sample and the tissues The results are expressed as µg of glucose 1 ¹⁴C con erted to ¹⁴C-carbon dioxide and ¹⁴C-

		Medium glucose (mg/ml)			
		0.025		0 100	
		Cells	Tissue	Cells	Tissue
CO	Basal	0.07 ± 0.01	0 04 ± 0.01	0.18 ± 0.02	0 11 ± 0.02
	Insulin (lmU/ml) l	0.90 ± 0 04	0.25 ± 0.03	2 73 ± 0 15	0 97 ± 0.12
Triglyc.	Basal	0.11 ± 0.01	0.06 ± 0.01	0.36 ± 0.02	0.13 ± 0.02
	Insulin (lmU/ml)	0.81 ± 0.06	0.21 ± 0.03	3 13 ± 0 11	0.69 ± 0.08

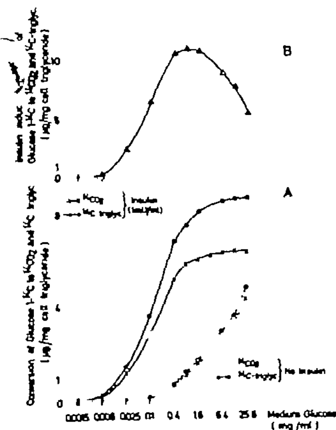


Fig. 2 A and B. The effec of in reasing extracell lar glucose con en rations on the metabolism of glucose l- ¹⁴C by isolated fat ll with and without insuli

Each lal contained 1 ml of medium nd 8.7 ± 0 5 mg of cell triglyceride (medium glucose 0.4—25 6 mg/ml) The ell suspension was diluted 1 3 when the medium contained less than 0 4 mg of glucose pe ml. Fig. 2 A show the conversion of glucose l- C t ¹⁴C-carbon dioxide and ¹⁴C-triglyceride w th and without insulin. Fig 2 B show the insulin-induced amount of glucose con erted to these tw parameters together

Each point represents the mean of 3 determinations.

and epididymal fat pads.
pads were halved and incubated in 2 ml of medium.
incubation 32—37 mg of triglyceride per sample.
triglyceride per mg cell triglyceride. Mean of 4 incubations ± S.D.

0.800		3.200	
Cells	Tissue	Cells	Tissue
1.21 ± 0.11	0.24 ± 0.04	1.83 ± 0.13	0.34 ± 0.03
4.28 ± 0.30	2.81 ± 0.47	4.37 ± 0.37	4.50 ± 0.71
0.97 ± 0.07	0.31 ± 0.08	1.90 ± 0.13	0.54 ± 0.11
3.51 ± 0.43	2.49 ± 0.40	5.73 ± 0.30	4.29 ± 0.37

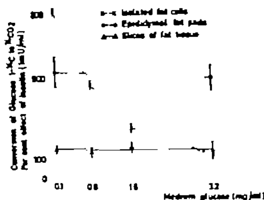


Fig. 3 The effect of glucose on the response to insulin of isolated fat cells, epididymal fat pads and slices of fat tissue.
Isolated cells were prepared from 8 fat pads and the paired pads were divided in halves and incubated. The slices were prepared from 8 fat pads. Isolated cells (6.3 mg of triglyceride) fat pads (45—62 mg of triglyceride) and slices of fat tissue (16—24 mg of triglyceride) were incubated in 2 ml of medium. The insulin concentration was 1 mU per ml.
The results are given as the effect of insulin in per cent of the basal activity. The points represent the mean of 4 slices ± S.D.

The following experiments were carried out in a ttempt to elucidate th nat re of the difference between the isolated cells and the intact tissue Free lls liberat d after treatment of the tissue with collagenase for 20—25 m n, were rec vered nd some were tudied immediately others after f rther treatment w th collagenase for p to 150 min No difference in the metabolism of glucose betw en these d ffer nt groups of cell could be detected.

Slices 0 3 mm of epididymal adipose tissue showed a h gher basal on ersion of glucose th h intact tissue or the free fat cells. Insulin nc eased the ^{14}C-carbon dioxide pr l ction about 1.0 per cent This ffect of nsulin or the sl es w independen t h oncentrations of glucose in the medium (0 1 mg ml to 3 2 mg ml) similar t t ct on the intact tissue and in nt ast to the ffect on olated cells (F g

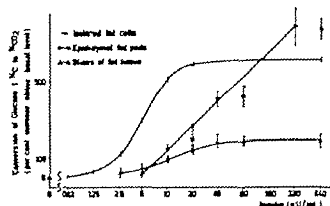


Fig. 4. The sensitivity to insulin of isolated fat cells, epididymal fat pads and slices of epididymal fat tissue.

Isolated fat cells were prepared from 8 fat pads. 16 pads were divided in halves and incubated. Isolated cells (8.4 ± 0.2 mg of triglyceride) and tissue segments (26—38 mg of triglyceride) were incubated in 2 ml of medium containing 0.5 mg of glucose. The slices (16—22 mg of triglyceride) were prepared from 8 fat pads on the following day and incubated similarly.

The results are expressed as per cent increase above the basal activity. The fat pad curve was drawn by connecting the points corresponding to 5 μU per ml and 320 μU per ml of insulin.

Each point represents the mean of 4 values ± S.D.

The sensitivity to insulin of free fat cells and epididymal fat pads.

Free cells were about 10 times more sensitive to insulin than the intact tissue (Fig. 4). Cells recovered as early as possible from the collagenase treated tissue and cells exposed to further treatment with collagenase for 150 min showed identical sensitivity to insulin. Slices of epididymal adipose tissue were more sensitive to insulin (in terms of the response to minimal concentrations of insulin) than the intact tissue but less sensitive than the free cells (Fig. 4).

The duration of the insulin effect on free fat cells and on epididymal fat pads.

Epididymal fat pads, which were incubated in a medium containing insulin in a concentration about 4 times that required for the maximal response and then washed, were fully stimulated during the following incubation period of 2 hrs. Isolated cells, preincubated in a similar manner and washed, were only stimulated for about 15—30 min. Addition of insulin antibodies had the same effect as the washing (Fig. 5). There was a time lag of about 15 min before a full insulin effect on the C-carbon dioxide production of the isolated cells was obtained, whereas the effect was immediate when glucose was added to cells preincubated with insulin in a glucose-free medium.

Discussion

Glucose metabolism

The effect of insulin (per cent increase above basal level) on the ^{14}C-carbon dioxide production from glucose-1-C by isolated fat cells depended on the extracellular concentration of glucose (Gliemann 1965 and 1967a). The present study shows quantitative differences between the free cells and the epididymal fat pads both in the glucose uptake and in the conversion of glucose-1-^{14}C to C-carbon dioxide and ^{14}C

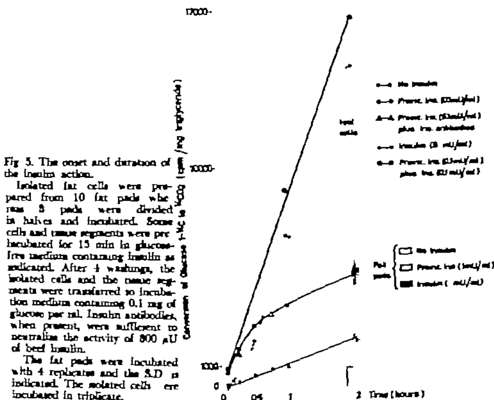


Fig 5. The onset and duration of the insulin action.

Isolated fat cells were prepared from 10 fat pads whe reas 8 pads were divided in halves and incubated. Some cells and tissue segments were pre incubated for 15 min in glucose-free medium containing insulin as indicated. After 4 washings, the isolated cells and the tissue segments were transferred to incubation medium containing 0.1 mg of glucose per ml. Insulin antibodies, when present, were sufficient to neutralize the activity of 800 μU of beef insulin.

The fat pads were incubated with 4 replicates and the S.D. is indicated. The isolated cells ere incubated in triplicate.

triglyceride. Two factors are of importance: (1) the glucose metabolism of insulin treated isolated cells reaches a maximal level t a lower medium glucose concentration than similarly treated "intact" tissue. As discussed below this may be due to the absence of a limitation of the diffusion of glucose from the medium and to the cell ( ) increasing glucose concentrations enhance the metabolism more in isolated fat cells than in "intact tissue also in the absence of insulin due to the disappearance of a barrier to glucose penetration into the cell.

The results on the carbon dioxide production and trigl ceride ynthesis f the n-tact tissue are in agreement with a previous report of Jeanrenaud nd Renold (1959) who found that the relativ effect of insulin was independent ro ghly f concentrations of glucose in the medium from 1 25 mM to 20 mM I the experiments of Rodbell (1964) the conversion of glucose U C to C-carbon dioxide nd C-fatty acids in the presence of insulin increased with rising concent tions of glucose in the medium until a concentration of about 0.5 mg of glucose per ml. In the absence of insulin, the glucose metabolism ncreased when the medium gluc se rose from 0.2 mg per ml to about 1.0 mg per ml. However in trast to the present experiments, no further increase took place nder these condition in response to still increasing concentrations of glucose

Crofford and Renold (1965) demonstrated that the diffusion of glucose into th epididymal fat tissue is rate limiting in the metabolism of glucose under certain con ditions (tissue from fed animals, relatively low glucose concentrations, presence o insulin) This explains that isolated fat cells utilize more glucose than fat pads when incubated in a medium with a low glucose concentration and insulin. It is also in agreement with the finding, that isolated fat cells metabolize slightly more gluc than the tissue when incubated in a medium containing a low concentration glucose and no insulin. This factor however cannot explain why the isolated c took up and metabolized 5 times more glucose than the tissue in the presence o high glucose concentration and in the absence of insulin. Under these conditio there was a barrier to glucose penetration into the fat cell of the intact tiss which was absent in the isolated fat cell. This barrier was apparently overcome treatment of the tissue with insulin.

Krahl (1961) has previously demonstrated that dividing of an epididymal fat p in smaller segments leads to an increase in the basal rate of glucose uptake and diminishing of the responsiveness to insulin. The slices of epididymal adipose tiss behaved similarly However the effect of insulin on these slices was independent the concentration of glucose in the medium (0 1—3 2 mg ml) This suggested th a difference between the adipose tissue and the free adipose cells was located close t the cell. If the difference was due to treatment with collagenase, the change had curred shortly after the cell was liberated from the tissue and no further alterati took place during a subsequent treatment with collagenase. In this connection, it is interest that fat cells of intact tissue are surrounded by a submicroscopic continuo basement membrane" (limiting membrane of the ground substance (Wasserma and McDonald 1960 which disappears after treatment with collagenase (Croffo *et al* 1966, Rodbell 1966)

(Autor and Lynn 1964 and Autor *et al.* 1965) found that isolated adipose c were completely permeable to glucose and postulated, therefore, that the effect insulin on the glucose metabolism of free fat cells was intracellular However t effect of insulin on their cells was moderate. Various factors in the preparation a incubation of the cells increase the basal glucose metabolism and thereby decre the insulin effect Gliemann 1967a) Hence, it seems possible that the cells stud by Autor and Lynn 1964 1965) had lost most of their insulin sensitivity Cro and Renold (1965 demonstrated that glucose transfer into the cell of the "inta adipose tissue is a major rate limiting step in glucose uptake under conditio adequate diffusion. Recently evidence was presented suggesting that this wa the case in the isolated fat cells (Crofford *et al.* 1966) supporting the hyp that a major action of insulin n the isolated fat cells, as well as on the fat cell of "intact" tissue, was to increase the glucose transfer They also found that the fre cells, in contrast to the cells of th intact" tissue, were partially permeable to tol (5 mM) This is in agreement with the present finding that glucose under tain conditions penetrated more asily into the isolated fat cells than into the fat c of "intact" tissue.

Sensitivity to insulin.

Most studies with epididymal fat pads, have indicated a response to 10—25μU of insulin per ml, the maximal effect being obtained with about 300—500 μU of insulin per ml (Martin, Renold and Dagenais 1958, Lyngsoe 1961 Sless, Telnzer and Wie and 1965) This is in agreement with the present results. In contrast, the isolated cells were sensitive to 0 63—1.25 μU per ml and an almost maximal effect was obtained with 20 μU per ml (Fig. 4) Rodbell (1964) found that isolated cells responded to insulin in the range of 10 μU per ml to 100 μU per ml. A previous study with a slightly different preparation method (Gliemann 1965) indicated a sensitivity range of 5 μU per ml to 500 μU per ml. A similar range was found by Blecher (1965) The marked differences in the sensitivity of isolated fat cells prepared by different groups are probably caused by differences in the preparation procedures. One important factor is to remove small amounts of collagenase adequately from the cell preparation (Gliemann 1967a)

Slices of epididymal f t pads were more sensitive (in terms of the response to minimal concentrations of insulin) than the "intact" tissue. This could be due to an easier diffusion of insulin into the slices. It is well established that rat adipose tissue rapidly inactivates insulin. Antoniades (1966) found that about half of the immunomeasurable insulin disappeared after incubation of fat pads (about 100 mg of tissue ml) with insulin (400 μU/ml) for 90 min. The partly purified cleavage system of 50 mg tissue converted 500 μU of insulin to TCA soluble material when incubated for 2 h. with 1 mU of insulin per ml (Di Girolamo *et al.* 1965) Experiments with isolated f t cells (10 mg of cell triglyceride/ml) showed no evidence of disappearance of crystalline insulin (10 μU/ml) after a 2 hrs incubation (Gliemann 1967b) suggesting that insulin is more rapidly inacti ated by the epididymal fat pad than by free fat cells. This might account for the difference in sensitivity together with a possible limitation of diffusion of the hormone into the tissue

Duration of the insulin effect

A high concentration of insulin in the interstitial fluid of the tissue may explain the difference between the duration of the insulin effect on isolated fat cells and epididymal fat pads. It is of interest in this connection that Garratt and coworkers (Garratt, Cameron and Menninger 1966, Garratt Jarrett and Keen 1966) have shown an association of ^{131}I labelled insulin to the diaphragm muscle over and above the quantity required to achieve maximal glucose uptake. They suggested that inactively bound insulin might serve as a depot

An apparent time lag on the insulin effect would appear if the isotope was diluted by unlabeled glucose present in the cells. However this was not the case in the present experiments since the basal con ersion of glucose increased linearly with time The time lag was caused by the process of insulin action rather than the subsequent glucose metabolism since th effect was immediate when the cells had been preincubated with insulin n glucose free medium.

In certain respects, the isolated fat cell appears to be a more unphysiological

preparation than the epididymal fat pad. A barrier to penetration of glucose and other small molecules such as sorbitol has disappeared. On the other hand, the direct contact of the free fat cells with the substrates of the bathing medium may be more similar to the in vivo supply through the capillaries than the long diffusion path through the fat pad. If so, the glucose metabolism of the fat tissue proceeds almost at its maximal rate throughout the normal range of plasma glucose concentrations if sufficient insulin is available. The high sensitivity of the cells to insulin is surprising since sera from these rats contain approximately 10 μU (fasted rats) to 80 μU (fasted refed rats) of suppressible insulin-like activity per ml (Gliemann 1967c). The in vivo sensitivity with insulin supplied through the capillary membrane, may therefore, be more similar to the in vitro sensitivity of the epididymal fat pad. The possible physiological significance of "depot" insulin is unknown.

Supported by Nordisk Insulinfond.

References

ANTONIADES, H. N. and S. N. GERSHOFF. Inhibitory effects of "bound" insulin on insulin uptake by isolated tissues. Diabetes 1966 15 655—662

AVRON, A. P. and W. S. LI. Insulin activity in isolated fat cells which are completely permeable to glucose. Biochem biophys R Comm 1964 17 80—85

AVRON, A. P., A. GUTMAN and W. S. L. Further observations on the nontransport effect of insulin. A N Y A ad S 1965 131 265—273

[illegible]OEHLER, H. U. and E. BERN. D[illegible] with glucose oxidase. I Methods of Enzymatic Analysis. Edit. H. U. Bergmeyer 1963 1?3—130

[illegible]LECHER, M. Phospholipase C and mechanisms of action of insulin and cortisol on glucose entry into free adipose cells. Bioch bi bioph R C mm 1965 21 202 209

CROFFORD, O. B. and A. E. RENOLD. Glucose uptake by incubated rat epididymal adipose tissue. Rate limiting steps and sit of insulin action. J biol Ch m 1965 240 14—21

CROFFORD, O. B., W. STAUFFACHER, B. JEANRENAUD and A. E. RENOLD. Glucose transport in isolated f t cells. Procedures for measurement of the intracellular w ter space. H h phys and pharmacol Act 1966 24 45—55

DI GIROLAMO, M., D. RODBELL, M. F. MALKIN and L. A. GARCIA. Inactivation of insulin by adipose tissue. Diabet 1965 14 87 92

DOLE, V. P. and H. MEINERTZ. Microdetermination of long-chain fatty acids in plasma and tissues. J biol Chem 1960 235 2595—2599

GARRATT, C. J., J. S. CAMERON and G. MENZINGER. The association of (131I) iodo-insulin with rat diaphragm muscle and its effect on glucose uptake. Bioch bi bioph A ta (Amst) 1966 115 179—186

GARRATT, C. J., R. J. JARRATT and H. KEEN. The relationship between insulin association with tissues and insulin action. Biochem. biophys Act (Amst) 1966 212 143—150

GLIEMANN, J. Insulin-like activity of dilute human serum assayed by an isolated adipose cell method. Diabete 1965 14 643—649

GLIEMANN, J. Assay of insulin-like activity by the isolated fat cell method. I Factors influencing the response to crystalline insulin. Diabetol gia 1967 I press

GLIEMANN, J. Unpubl observ. 1967b

GLIEMANN, J. Assay of insulin-like activity by the isolated fat cell method. III Studies on the non-suppressible ILA of serum. Diabetologia 196 I press

JEANRENAUD, B. and A. E. RENOLD. Studies on rat adipose tissue in vitro. IV Metabolic patterns produced in rat adipose tissue by varying insulin and glucose concentrations independently from each other. J biol Chem 1959 234 3082 3087

KRAHL, M. E. The action of insulin. Academic Press Inc. New York and London. 1961 p. 183

L[illegible], J. Determination of the insulin-like activity in serum using an epididymal adipose tissue. S nd J cli Lab I 1961 13 628—636

Martin, D. B., A. E. Renold and Y. N. Dagenais, An assay for insulin-like activity using rat adipose tissue. *Lancet II* 1958, 6—77

Rodbell, M., Metabolism of isolated fat cells. I. Effect of hormones on glucose metabolism and lipolysis. *J. biol. Chem.* 1964, *239* 375—380

Rodbell, M., Metabolism of isolated fat cells. II. The similar effects of phospholipase C and of insulin on glucose and amino acid metabolism. *J. biol. Chem.* 1966, *241* 130—139

Siess, E., A. Teinzer and O. Wieland, Eine vereinfachte Fettgewebsmethode zur Insulinbestimmung im Serum. *Diabetologia* 1965 *1* 201—207

Wassermann, F. and T. F. McDonald, Electron microscopic investigation of the surface membrane structures of the fat cell and of their changes during depletion of the cell. *Z. Zellforsch. Abt. Histochem.* 1960, *52* 778—800

of the duodenal stump. Two to three weeks were allowed for recovery.

The dogs were fasted for 18—24 hrs before each experiment. In control experiments the secretory outputs of acid and pepsin from the Pavlov pouches in response to sham feeding for 10 min with ground beef, and to i.v. infusion of gastrin or histamine for 4 1/2 hrs were determined. The low volume response following sham feeding did not allow pepsin determinations in 15-min samples. Therefore, acid and pepsin outputs in the control experiments are expressed as 2-hrs values.

The effect of three different doses of gastrin and histamine on the secretory response to sham feeding was determined as follows. The secretory response to intravenous infusion of gastrin or histamine was followed with 15-min samples for 2 hrs. After this period the secretion had usually reached steady state. At this point the dogs were sham fed and the response to the combined stimulation by sham feeding and by the intravenous stimulant was followed for 2 1/2 hrs. Animals were not used on any date that their spontaneous secretion exceeded 0.02 mEq/15 min as determined by 4 collection periods prior to sham feeding or before the infusion of gastrin or histamine was started.

The volumes of the gastric samples were measured and they were analyzed for acid by titration with 0.01 N NaOH using phenolphtalein as indicator. Pepsin was determined with the hemoglobin substrate method (Bucher, Grossman and Ivy 1945). Histamine dihydrochloride was infused in doses of 0.125, 0.5 and 1.0 mg/hr. Gastrin, prepared according to the method of Gillespie and Grossman 1963) was infused in doses corresponding to 1.25 10.0 and method of Gillespie and Grossman (1963) was infused in doses corresponding to 1.25 10.0 and the same material. The stimulants were dissolved in physiological saline and were given as constant intravenous infusion at a rate of 12 ml/hr.

Results

The mean 2 hrs acid and pepsin outputs to sham feeding or to intravenous infusion of three different suprathreshold doses of gastrin or histamine were *determined in* 3 antrectomized dogs with 3—4 expts. on each dog for every type and dose of stimulus (Table I). The doses of gastrin and histamine were choosen *to range from* a just suprathreshold dose for acid secretion (Table I) to a dose producing 50—75 per cent of maximal acid secretion. In these dogs which had their *gastrin releasing* regions—the antrum and the duodenal bulb—removed, the acid response to sham feeding was very low (Table I).

TABLE I

Stimulus	Acid output mEq/2 hrs	Pepsin output P.U.^Hb/10^4/2 hrs
Sham feeding	0.32 ± 0.03	7144 ± 1120
Histamine alone		
0.125 mg/hr	0.48 ± 0.06	1504 ± 230
0.5	3.52 ± 0.41	1301 ± 198
1.0	6.48 ± 0. 1	2290 ± 340
Gastrin alone		
1.25 μg/h	0.32 ± 0.0.	1640 ± 330
10.0	2.24 ± 0.62	744 ± 140
20.0	3.52 ± 0.51	944 ± 210

Mean acid and pepsin outputs ± S.E. in response to sham feeding, histamine and gastrin in 3 antrectomized dogs with 3—4 expts. on each dog for each type and dose of stimulus. In the histamine and gastrin infusion experiments all values has been taken from the second period.

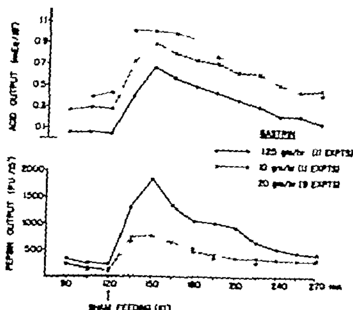


Fig. 1. Mean acid and pepsin responses to sham feeding during i.v. infusion of different suprathreshold doses of gastrin in 3 antrectomized Pavlov pouch dogs with 3—4 expts. on each dog for each dose.

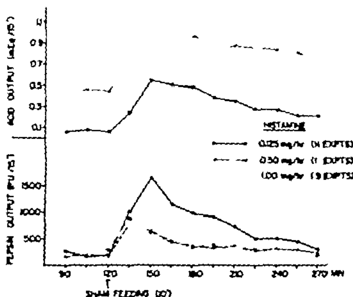


Fig. 2. Mean acid and pepsin responses to sham feeding during i.v. infusion of different suprathreshold doses of histamine in 3 antrectomized Pavlov pouch dogs with 3—4 expts. on each dog for each dose.

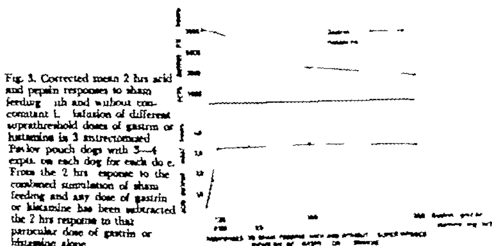


Fig. 3. Corrected mean 2 hrs acid and pepsin responses to sham feeding with and without concomitant i. v. infusion of different suprathreshold doses of gastrin or histamine in 3 antrectomized Pavlov pouch dogs with 3—4 expts. on each dog for each dose. From the 2 hrs response to the combined stimulation of sham feeding and any dose of gastrin or histamine has been subtracted the 2 hrs response to that particular dose of gastrin or histamine alone.

Sham feeding alone was found to be a more potent stimulus of pepsin secretion than gastrin or histamine (Table I). The pepsin responses to different doses of gastrin or histamine showed no significant ($p>0.05$ according to the Student t-test) differences (Table I).

All three doses of gastrin and histamine caused a considerable increase of the acid sham feeding response (Fig. 1 and 2, top curves). In order to estimate the degree of augmentation produced by the different doses of gastrin and histamine the following calculations were made. The 2 hrs acid output in response to gastrin or histamine alone was subtracted from the 2 hrs acid response to combined stimulation with sham feeding and gastrin or histamine. In this way it could be shown that all doses of gastrin and histamine increased the sham feeding response to the same extent (Fig. 3). Even with threshold doses of the two humoral stimuli the potentiating effect on sham feeding response was maximal.

The pepsin response to sham feeding during gastrin or histamine infusion decreased in every 15-min period with the larger doses of either agent (Fig. 1 and 2, bottom curves). The sham feeding induced 2 hrs pepsin output was not significantly influenced by the concomitant infusion of low doses of gastrin (1.25 gm/h) or histamine (0.125 mg/hr). High doses of the humoral stimuli caused a significant depression of the sham feeding pepsin response ($p<0.05$ according to the Student t-test). The results were not changed if the 2 hrs pepsin output in response to gastrin or histamine alone was subtracted from the 2 hrs response to sham feeding combined with gastrin or histamine (Fig. 3).

Discussion

In Pavlov pouch dogs with their gastrin releasing areas removed the acid gastric response to sham feeding has been shown to be potentiated by sub-threshold doses of gastrin or histamine (Olbe 1964 a and 1966). The potentiating effect of gastrin was proportional to the dosage. In the present study it was shown that also supra-

threshold doses of gastrin and histamine augmented the sham feeding acid response. The acid response to the combined stimulation by sham feeding and gastrin or histamine increased with increasing suprathreshold dose of either agent (Fig 1 and 2) which might give the impression of a greater augmentation of the sham feeding response with suprathreshold doses gastrin or histamine than with threshold doses. However the increase of the acid response to the combined stimulation with suprathreshold doses of gastrin or histamine corresponded always to the acid output that the suprathreshold dose of either agent produced when given alone. This increase can thus be completely explained as due to additive effects of suprathreshold doses of gastrin or histamine on the sham feeding response already potentiated by threshold doses of these humoral stimuli (Fig. 3) In other words the potentiating effect of gastrin and histamine on the acid secretory response to vagal activation reaches its maximum already at threshold doses of these stimuli. This fact emphasizes the important role which low plasma levels of gastrin (and histamine?) may play in the physiological control of gastric acid secretion.

Sham feeding alone produced as much pepsin as did sham feeding combined with a threshold dose for acid secretion of gastrin or histamine.

We are well aware of the unexplored possibility that subthreshold doses of gastrin or histamine may augment the pepsin response to sham feeding On the other hand suprathreshold doses of gastrin or histamine significantly suppressed the pepsin ponse to sham feeding. As regards histamine, this finding confirms an old ob- on of Alley (1935) Gillespie and Grossman who used combined stimulation Urecholine and gastrin or histamine found inhibition of the pepsin response in h in pouch dogs with a high dose of Urecholine (Gillespie and Grossman 1964) and they were also able to show a potentiated pepsin response in Heidenhain pouch dogs but not in Pa lov pouch dogs with low doses of Urecholine (Passaro and Grossman 1964)

Passaro and Grossman 1964 found that Gillespie and Grossman gastrin extract stimulated sustained pepsin secretion from the innervated stomach in dogs only when given in low doses—i.e 1 25 g hr which is the same dose as the threshold dose for acid secretion in the present study Emås and Grossman (1967) found that Gregory and Tracy gastrin extract in the whole submaximal dose range for acid secretion stimulated pepsin secretion in dogs and cats with gastric fistula. This finding has been confirmed by Cooke and Grossman (to be published) using pure swine gastrin and synthetic human gastrin. Thus it appears that pure gastrin does stimulate pepsin secretion while impure gastrin extracts may not stimulate pepsin secretion over the whole dose range for acid secretion. The finding of the present study that a threshold dose of Gillespie and Grossman gastrin extract (1 25 g hr) did not interfere with the pepsin response to sham feeding seems reliable in spite of the use of an impure gastrin extract, since this dose according to Passaro and Grossman (1964) produces pepsin secretion (cf Table I where however the differences between the pepsin outputs to various doses of gastrin are not statistically significant The finding of the present study that submaximal doses of gastrin extract inhibited

the pepsin response to sham feeding should, however be cautiously interpreted because of the possible pharmacological action of crude gastrin, although this inhibition is not a characteristic of impure gastrin but is also produced by histamine

Our present concepts of the neuro-humoral mechanism for stimulation of gastric secretion can be summarized in the following way

The interaction between neural and gastrin mechanisms has for a long time been suggested to be essential for stimulation of acid secretion (U[illegible] 1942 and Olbe 1964 a and b) but does not seem to play a major role in the pepsin secretory response. Vagal activation, within physiological limits, by direct cholinergic action on the gastric glands *suffices to produce pepsin secretion but requires the presence of* gastrin to produce significant acid secretion. The addition of small amounts of gastrin—up to the threshold dose for acid production—does not seem to interfere with the direct vagal action on the pepsin secreting glands but potentiates the vagal action on the acid secreting glands. Further increase in the dose of gastrin inhibits the vagal action on the pepsin secreting glands, and has only an additive effect on the already potentiated acid response. The conclusions are drawn with due reservation for possible species and other differences between the gastrin preparations available at present.

This investigation was supported by Torsten och Ragnar Söderbergs Stiftelse, Stiftelsen The[illegible] och Johan Anderssons Minne, Gustaf och Thyra Svenssons Minne and The Swedish *Medical* Research Council (17X-88-01)

References

ALLE[illegible] A., The inhibitory effect of histamine on gastric secretion. *Amer J [illegible]* [illegible] 787—794.

B[illegible], G. R., M. I. GROSSMAN and A. C. I[illegible] A pepsin method. The [illegible] determination of peptic activity. *Gastroenterology* 1945 5 301—311

COOKE, A. R. and M. I. GROSSMAN, T[illegible] be publ.

EMÅS, S and M. I. GROSSMAN, Effect of truncal vagotomy on acid and [illegible] histamine and gastrin in dogs. *Amer J Physiol.* 1967 *212* 1007—101[illegible]

GILLESPIE, I. E. and M. I. GROSSMAN, Inhibition of gastric secretion by [illegible] gastrin. *Gastroenterology* 1963 *44* 301—310.

GILLESPIE, I E. and M. I GROSSMAN. Potentiation between U[illegible] and between Urecholine and histamine in stimulation of Heidenhain [illegible] 71—76.

OLBE, L., Esophageal cannula dog, a simple mode of preparation [illegible] ments. *Gastroenterology* 1959 *37* 460—462.

OLBE, L. Significance of vagal release of gastrin during the [illegible] in dogs. *Gastroenterology* 1963. *44* 463—468

OLBE, L., Potentiation of sham feeding response in Pavlov [illegible] of gastrin [illegible] and [illegible] acidification of the [illegible] 1964 a. *61* [illegible]—234

OLBE, L. Effec[illegible] of resection of gastrin releasing region [illegible] and insulin hypoglycemia in Pavlov pouch dogs. *Acta p*[illegible]

OLBE, L. Potentia[illegible] of acid sham feeding response in Pa[illegible] by mesenteric [illegible] anastomosis. *Acta physiol. scand* [illegible]

PASSARO, E P and M I. GROSSMAN Effect of [illegible] to histamine and gastrin. *Amer J. Physiol.* 196[illegible] [illegible] 173 [illegible]

THOMAS, J E. A simplified procedure for prepar[illegible] Pavlov pouch [illegible] *p. Biol* (N Y) 194[illegible] *50* 58—61

U[illegible], B The part played by the pyloric [illegible] phase of [illegible] *Act physiol scand* 194[illegible] Suppl. 13

Acta physiol. scand. 1968. 72. 498—509

From the Institute for Experimental Medical Research, University of Oslo, Ullevål Hospital, Norway

Effect of Adrenaline, Noradrenaline, Angiotensin and Renal Nerve Stimulation on Intrarenal Distribution of Blood Flow in Dogs

By

KNUT AUKLAND

Received 24 August 1967

Abstract

AUKLAND, K. *Effect of adrenaline, noradrenaline, angiotensin and renal nerve stimulation on intrarenal distribution of blood flow in dogs.* Acta physiol. scand. 1968. 72. 498—509.

The acute effect of adrenaline, noradrenaline, angiotensin and renal nerve stimulation on intrarenal blood flow was studied in 15 dogs. Local clearance of hydrogen gas measured polarographically with 2—4 needle shaped platinum electrodes in the outer medulla served as indicator for medullary blood flow. Total renal blood flow was measured simultaneously with electromagnetic flowmeter. Adrenaline, noradrenaline and angiotensin infused i.v. or into the renal artery over a period of 5—15 min reduced outer medullary hydrogen clearance and total renal blood flow to 25—90 per cent of control and on an average to the same extent. Renal nerve stimulation produced the same pattern with flow reduction to 50—75 per cent of control. Since changes in total renal blood flow mainly reflect changes in cortical perfusion, it is concluded that resistance to flow was increased proportionately in the cortical and juxtamedullary circulation. Considerable local variations in response of outer medullary hydrogen clearance observed with all stimuli suggested local differences in [illegible] response but no consistent difference between cortex and medulla.

The acute effect of adrenaline, noradrenaline, angiotensin and renal nerve stimulation is to increase renal vascular resistance. In dogs, angiotensin and renal nerve stimulation may reduce blood flow to 25—50 per cent of control, whereas catecholamine injections may cause complete cessation of renal blood flow. In the latter case it is obvious that the whole kidney becomes ischemic, but it is conceivable that smaller doses could produce selective ischemia in parts of the kidney or at least a change in the normal intrarenal flow distribution. Such a response type was proposed by Trueta [illegible] al. (1947) who reported that adrenaline infusion, crush injuries and nerve stimulation in rabbits often caused complete cortical ischemia while the blood was diverted through the juxtamedullary circulation. The same response was later observed after renin injection (Daniel, Prichard and Ward-McQuaid 1954). This conclusion was based on qualitative methods, such as India ink injec-

ons and angiography Among the numerous investigators repeating Trueta's experiments with similar methods, some confirmed and others denied the existence of the "Trueta shunt" and several authors emphasized the difficulties of producing a medullary diversion in the dog. More quantitative methods, such as local heat clearance (Scher 1951 Sadler and Tuttle 1963) heat appearance time (Ochwadt and Schmier 1954) PAH extraction (Reubi and Schroeder 1949) and externally recorded Krypton[85] clearance (Carriere *et al.* 1966) have also given conflicting results.

In the present study renal medullary blood flow was estimated from local clearance of hydrogen gas measured polarographically with needle-shaped platinum electrodes placed in the outer medulla of anesthetized dogs. It was found that infusions of adrenaline, noradrenaline and angiotensin, as well as renal nerve stimulation, on average reduced outer medullary hydrogen clearance to the same degree as total renal blood flow suggesting a roughly parallel reduction of cortical and medullary blood flow

Methods

Experiments were performed in 13 mongrel dogs weighing 15—28 kg. Anesthesia was initiated with intravenous injection of Nembutal, 25 mg/kg, and maintained with subsequent doses of 2—3 mg/kg. A tracheal tube was inserted to secure free airways, but artificial respiration was not used. A polyethylene catheter was inserted into femoral or brachial artery for blood sampling and for recording mean arterial pressure by means of Statham transducer and Sanborn amplifier and recorder In six experiments sodium para-aminohippurate (PAH) and creatinine in 0.9 % saline was given as constant intravenous infusion at rate of 2 ml/min, providing steady plasma concentrations of PAH of 1 to 2 mg/100 ml and creatinine of 10 to 20 mg/100 ml. Two to four clearance periods of 10 min duration were conducted during control conditions in each experiment. PAH was determined in plasma and urine by the method of Smith *et al.* (1945) and creatinine according to Bonsnes and Taussky (1945) Effective renal blood flow" was calculated as PAH-clearance/(1—hematocrit)

The right or left kidney was exposed retroperitoneally through flank incision. The ureter was cannulated with polyethylene catheter and polyvinyl catheter was introduced into the renal artery ad modum Herd and Barger (1964) the tip directed outwards. An electromagnetic flowmeter probe (Nycotron, Oslo) was placed on the renal artery proximal to the catheter and nylon snare round the artery was used for checking the zero reading on the flowmeter The flowmeter had been directly calibrated on femoral or carotid arteries of the same calibre

Two to four needle-shaped platinum electrodes were inserted into the outer medulla, usually from the medial or lateral aspect of the kidney as shown in Fig. 1 (Ea) and the L-shaped electrode shaft was fixed to the renal capsule by silk suture. The length of the active platinum surface at the pointed electrode tip was 1—1.5 mm, and the diameter at the base about 0.5 mm The kidney was then *returned to normal* position, and the wound temporarily closed.

Hydrogen oxidation current was measured in principle as described by Aukland, Bower and Berliner (1964 A constant polarizing potential of +0.2 Volt versus KCl-saturated calomel half-cell was provided by an operational amplifier (Fig. 1) Six such amplifier units connected to Rikadenki potentiometer recorder (Rikadenki Kogyo Co., Tokyo, Model B-64) permitted simultaneous measurements from six electrodes.

Hydrogen gas was blown into the tracheal tube at rate giving an arterial concentration of 5—10 per cent of full saturation. Hydrogen inhalation was discontinued when the hydrogen current from the electrodes in the outer medulla had levelled off, usually after 5 to 10 minutes. At the same time, manual infusion of hydrogen-saturated saline into the renal artery was started at rate adjusted to maintain electrode current approximately unchanged for an additional period of 1—2 min. This procedure allowed time for hydrogen to be removed from systemic arterial blood, and provided an almost instantaneous desaturation of renal arterial blood when the infusion was stopped.

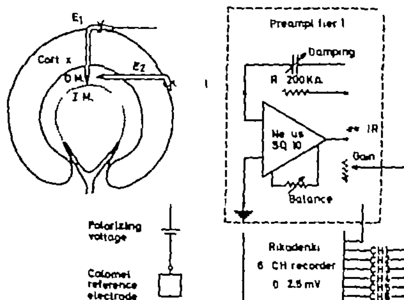


Fig. 1 Six-channel polarograph for hydrogen gas determination. Position of platinum electrodes (E and E) in outer medulla shown on kidney cross section. O M. = outer medulla, I. M. = inner medulla.

g dm ust ation Adrenaline noradrenaline and angiotensin in isotonic saline were given or into the renal artery by means of an electric infusion pump at rate of 2—6 ml/min infusion was started before or after loading the kidney with hydrogen gas, but in each 2—8 min were allowed before starting desaturation By this time steady state had ully been reached, judging from constan flowmeter reading but occasionally the infusion rate had to be increased to maintain constant renal blood flow during desaturation. Drug infusion rates were as follows Adrenaline, 12 µg/min and 0.6—3 µg/min noradrenaline 5—30 µg/min i and 0.6—3 5 µg/min a. and angiotensin 0 5 1.2 µg/min i.v and 0.04—0.06 µg/min a. Most experiments included 3 —4 experimental periods w h control periods before and after

R al stim latio In fi experiments the renal nerves were dissected free and hooked onto bipolar platinum electrode Usually 5—6 major nerves were isolated, pproaching he hilus in caudal direction Tiny nerv branches surrounding the renal art ry were difficul to free but were included as far as possible A Grass electrical stimulator was used, gi ing squar wave pulses of 1 msec duration. Stimulation w th 5—15 Volt t ate of 3 0 ps as *started —10 min before ecording medullary hydrogen desaturation I some periments* blood flow tended to increase despit constant stimulation, and the stimulation frequency therefore had to be slightly increased to maintain constant total renal blood flow during desaturation.

In one dog, electrodes and renal artery atheter wer left in place for studies without anesthesia tw and four da s later At the end of all other expe iments, he kidney was ex cised, the lectrodes arefully dissected out and their position measured as the distanc from the cortico-medullary borde Of 57 lectrodes tentativel inserted int the outer medulla, 10 had to be excluded for the following reasons 4 lectrodes were placed in the corte or on the cortico-medullary border 5 were in the outer medulla, bu in lose ontact w h ah es or larger arteries or eins, and 1 lectrode was defectiv After removing the lectrodes and perirenal fat, the kidney was weighed to the nearest gram.

Results

Control measurements Each experimental period was preceded and followed by a control period, and the experimental values will be related to the average of these

controls. Renal blood flow measured with electromagnetic flowmeter in 13 expts. averaged 218 ml/min (range 150—320 ml/min) in the experimental kidney. The flow per gram kidney weight after dissecting out the electrodes (15—30 per cent weight loss) ranged from 3.5 to 5.8 ml/min, with an average of 4.8 ml/min/gram. Effective renal blood flow" estimated from PAH clearance in 4 expts., was 14—28 per cent lower than the flowmeter values, corresponding to an average PAH extraction of 80 per cent. Glomerular filtration rate, measured in 6 expts. as creatinine clearance, averaged 40 ml/min (range 30—56 ml/min). Urine flow was usually 0.2 to 0.5 ml/min, and in no experiment exceeded 1.5 ml/min.

The hydrogen desaturation curves from the outer medulla showed the same characteristics as those described previously (Aukland 1967a) i.e. increasing initial delay and decreasing desaturation rate going from the cortico-medullary border towards inner medulla. The clearance constant, k, indicating the fraction of hydrogen gas removed per minute, was calculated from the half time ($T_{1/2}$) of the first exponential part of the desaturation curve as $k = 0.693/T_{1/2}$ (Fig. 2, left). The half time was measured in minutes, and the clearance constant has accordingly the dimension min^{-1}. In the deepest part of the outer medulla, clearances of 0.2 min^{-1} were observed, whereas values up to 2.0 min^{-1} were measured close to the cortico-medullary border. Most of the 47 accepted electrodes were placed approximately in the middle of the outer medulla (2—4 mm from cortex) giving an average control clearance of 0.54 min^{-1}.

Effect of catecholamines. In preliminary experiments, it was shown that rapid intravenous injection of 5—10 μg adrenaline or noradrenaline during hydrogen desaturation caused a marked reduction of the slope of the desaturation curves, followed by return towards control in the course of 1—3 min. Since this procedure did not allow quantitative estimate of the response, the drugs were continuously infused to provide a steady state in further experiments in 10 dogs. In all, 18 experimental periods were run, including clearance measurements from a total of 31 electrodes in the outer medulla.

Fig. 2 shows original desaturation curves obtained from 3 electrodes in the outer medulla, situated 2—4 mm from the corticomedullary border. The left part of the figure shows a control run which gave fairly good monoexponential curves from all electrodes, as evident from the semilogarithmic plot below the original tracing. The kidney was again loaded with hydrogen, and i.v. infusion of noradrenaline 10 μg/min, was begun. Four minutes later when the outer medullary hydrogen current curve had levelled off hydrogen administration was stopped, the infusion of noradrenaline continuing for another 5 min. The desaturation rates were now considerably reduced compared to control, although to varying extents at the different electrode sites (Fig. 2, right). It is also clear, especially from the semilogarithmic plot, that the desaturation rate increased rapidly after discontinuing noradrenaline infusion. Total renal blood flow was not measured in this experiment.

The effect of i.v. infusion of adrenaline is further illustrated in Fig. 3. After two control periods, showing slightly rising renal blood flow and hydrogen clearances

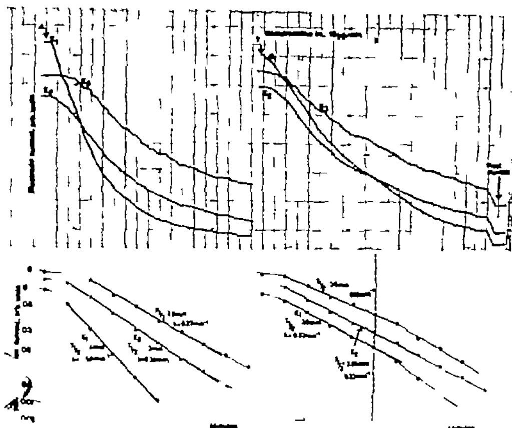


Fig. 2. Original hydrogen desat ration curves from three lectrodes in the middle of outer medulla i control period (left) and during infusion of noradrenaline (right). In semi logarithmic plot (low part) arbitrary current units hav been chosen to avoid overlapping of curves.

from four electrodes in the outer medulla, adrenaline was given i.v. at a rate of 12 µg/min during the next clearance period. Mean arterial pressure rose by about 50 mm Hg, whereas total renal blood flow and medullary clearances were reduced by approximately 30 per cent. A subsequent control run showed good return to control levels for both total flow and local medullary clearances.

In order to compare different experiments, clearance for each electrode during catecholamine infusion was calculated in per cent of average pre- and post-experimental control. All experimental periods with simultaneous measurements with electromagnetic flowmeter are shown in Fig. 4 where total renal blood flow is also given in per cent of control. With intravenous infusion both adrenaline and noradrenaline considerably increased mean arterial pressure from 10—60 mm Hg. Outer medullary clearances often fell to varying degrees at the different electrode sites but on average in proportion to the fall in total renal blood flow. Both parameters remained almost unchanged in three infusion periods, but since arterial pressure increased these observations also indicated increased vascular resistance

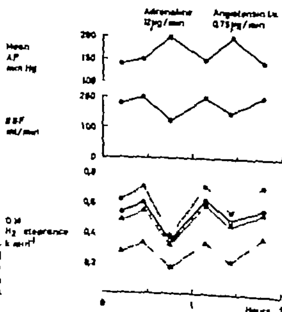
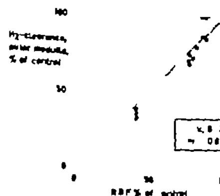


Fig. 3 Effect of i.v. infusion of adrenaline and angiotensin on mean arterial pressure, total renal blood flow (RBF) and hydrogen clearance measured with four electrodes in outer medulla (O.M.)



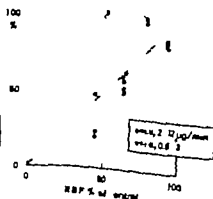


Fig. 4 Effect of noradrenaline and adrenaline on outer medullary hydrogen clearance in per cent of control for each electrode related to total renal blood flow in per cent of control

With intra-arterial infusion of catecholamines, arterial pressure remained unchanged or rose by less than 10 mm Hg. Total renal blood flow fell markedly even with doses of 0.6 µg/min, and outer medullary hydrogen clearance was reduced approximately the same extent for most electrode sites. However two electrode sites showed practically no change of clearances during intra-arterial adrenaline infusion (Fig. 4). This was probably due to insufficient mixing of adrenaline with renal arterial blood, as these electrodes also failed to respond to infusion of hydrogen-saturated saline into the renal artery catheter. These observations have nevertheless

TABLE I Effect of noradrenaline (NA) infusion on outer medullary hydrogen clearance measured with four electrodes (E_1—E_4) in unanesthetized dog, two and four days after electrode implantation

	E_1, min^{-1}	E_2, min	E_3, min^{-1}	E_4, min^{-1}
2nd day				
Control	0.31	0.37	0.43	0.39
NA i.v. 50 μg/min	.20	.36	41	.26
NA i.a., 6 μg/min	.08	.07	13	18
4th day				
Control	42	41	43	..8
NA i.v., 15 μg/min	.30	.31	41	46
NA i.a., 1.5 μg/min	16	16	.28	40

been included for comparison with total renal blood flow since it must be assumed that areas of the cortex also failed to receive adrenaline. Average of percentual clearances for each infusion period are shown in Fig. 7 illustrating the similar response of the medullary and the total renal circulation to catecholamine infusion.

The effect of noradrenaline, infused i.v. or into the renal artery in an unanesthetized dog with chronically implanted electrodes is shown in Table I. Clear fell in all infusion periods, except at one electrode site (E_3) which showed no ificant change during i.v. infusion of noradrenaline. Arterial pressure and total blood flow were not measured in these experiments.

Effect of angiotensin. Angiotensin was given in 5 expts. in doses about one tenth of those used for catecholamines. In the example shown in Fig. 5 infusion of 0.75 μg/min i.a. caused a marked rise in mean arterial pressure while total renal blood flow and outer medullary hydrogen clearance measured simultaneously with four electrodes decreased to about 75 per cent of control. Similar results were obtained in 6 additional experimental periods, including clearances from 13 different electrode positions. As evident from Fig. 5 the percentual decrease of clearances was usually of the same magnitude as for total renal blood flow. Clearance for one electrode did not respond to i.a. infusion, again presumably due to inadequate mixing in the renal artery and failure of angiotensin to reach all areas of the kidney. However when simultaneous clearances are averaged, as in Fig. 7 they show a reduction closely parallel to total renal blood flow indicating a similar rise in juxtamedullary and total renal resistance.

Renal nerve stimulation. The effect of renal nerve stimulation was studied in 5 animals with a total of 11 stimulation periods with control measurements before and after. Total renal blood flow decreased in all stimulation periods. Mean arterial pressure remained unchanged or increased transiently by 5—10 mm Hg in some stimulation periods. Outer medullary hydrogen clearance, measured at 18 different electrode sites, usually decreased substantially during stimulation, but to varying

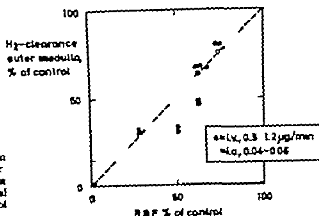


Fig. 5. Effect of angiotensin on outer medullary hydrogen clearance in per cent of control for each electrode, related to total renal blood flow in per cent of control.

degrees (Fig. 6). In one experiment, a major renal nerve slipped off the stimulating electrode, resulting in complete abolition of response to stimulation on hydrogen clearance measured from two electrodes in the lower half of the kidney whereas two electrodes in the upper half still showed marked reduction of clearances. Although these experimental periods have been excluded from the results, it seems likely that lack of response at two additional electrode sites (Fig. 6) might also be due to failure to include all renal nerves in the stimulating electrode, or to failure to stimulate all nerves within the fairly thick bundle hooked up on the electrode. However average clearance from all electrode sites in each experimental period decreased to practically the same extent as total renal blood flow (Fig. 7).

Discussion

The experiments showed that adrenaline, noradrenaline and angiotensin, whether injected i.v. or into the renal artery reduced outer medullary hydrogen clearance

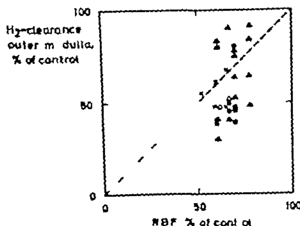


Fig. 6. Effect of renal nerve stimulation on outer medullary hydrogen clearance in per cent of control for each electrode, related to total renal blood flow in per cent of control. Each experiment represented by one symbol.

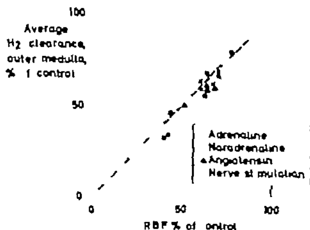


Fig. 7 Percentual outer medullary hydrogen learance averaged for each experimental period, related to total renal blood flow in per cent of control

largely to the same extent as total renal blood flow and the same pattern was produced by electrical stimulation of the renal nerves. Since the cortex constitutes about 70 per cent of the kidney and probably receives an even larger fraction of renal blood flow total renal blood flow will mainly reflect flow through the cortex. Provided that outer medullary hydrogen clearance reflects medullary blood flow it follows that catecholamines, angiotensin and renal nerve stimulation reduce medul- y and cortical blood flow to approximately the same extent or in other words, ad to a roughly parallel rise in resistance to blood flow in the cortical and the stamedullary circulation.

The use of hydrogen clearance from the outer medulla as an indicator for medullary blood flow deserves comment Whereas urine flow may contribut considerably to gas clearance from the inner medulla, the influence of changes in urine flow of 1—? ml min or less on outer medullary clearance is negligible (Aukland and Berliner 1964) Since gas removal by diffusion must also be negligible (Aukland 1967a) it follows that outer medullary hydrogen clearance must mainly be a function of medullary blood flow However due to the counter current exchange of gas between ascending and descending vasa recta, the clearance is not necessarily linearly related to blood flow According to a formula developed by Berliner et al (1958) and Günzler (Thurau and Deetjen 1962) clearance should be proportional to (Flow) where $1<n<2$. Unfortunately the determinants of n are unknown but even the most extreme deviation from proportionality i.e. n=2 would not invalidate the present conclusion that medullary blood flow was reduced approximately in proportion to cortical flow This uncertainty however and the problem of defining local blood flow in the renal medulla, prohibit conversion of clearances to absolute blood flow (Aukland 1967a)

The considerable scatter of response at different electrode sites also with uv infusions (Fig. 4—6) suggests that the constriction of the juxtamedullary resistance vessels may be of varying intensity in different segments of the kidney A similar pattern may well be produced in the cortex. It should be emphasized that the present

[illegible] is based on infusion and stimulation periods of only 5—15 min duration, and should not be extended to more chronic stimuli without further experiments.

None of the present experiments revealed any evidence for a "juxtamedullary diversion of cortical blood flow" as observed in rabbits by Trueta *et al.* (1947). It should be noted, however, that several other distribution patterns were described by Trueta, but have been overlooked by most later reviewers. While confirmed by some, other investigators failed to reproduce Trueta's observations, and also pointed out the shortcomings of the injection technique (Kahn, Skeggs and Shumway 1950). As there seem to be definite species differences in response type, the following discussion will mainly deal with experiments in dogs. Trueta's former collaborators, Daniel, Peabody and Prichard (1952) succeeded in producing medullary diversion with adrenaline in only one out of several dogs, whereas Moyer *et al.* (1950), Houck (1951a) and Christensen (1952) failed to produce this pattern. No injection studies seem to have been made in the dog during infusion of noradrenaline or angiotensin, whereas two groups reported normal distribution of India ink in rabbits with the latter agent (Hughes-Jones et al. 1949, Kahn et al. 1950). Several investigators pointed out that direct or indirect nerve stimulation caused a great variety of distribution patterns, such as pyramidal, segmental or patchy ischemia both in cortex and medulla, but not selective cortical ischemia (Goodwin, Sloan and Scott 1949, Moyer *et al.* 1950, Study and Shipley 1950, Houck 1951b, Block, Wakim and Mann 1952a).

Scher (1951), using the heated thermocouple principle, found reduction of heat conductivity in both cortex and medulla during infusion of adrenaline and noradrenaline as well as during renal nerve stimulation, and concluded that cortical and medullary blood flow was reduced to approximately the same extent, in agreement with the present results. On the other hand, Sadler and Tuttle (1963) found more pronounced reduction of cortical than of medullary heat clearance during noradrenaline infusion, but since medullary heat clearance is to a great extent determined by diffusion and not by blood flow (Aukland 1967b), the clearances cannot be directly interpreted in terms of local blood flow. This difficulty with heat as indicator may [illegible] explain the finding of a greater prolongation of cortical than of medullary [illegible] appearance time during adrenaline infusion (Ochwadt and Schimer 19[illegible]).

The re[illegible] of PAH or Diodrast has also been used to evaluate the distribution [illegible] blood flow. A[illegible] that these substances are not [illegible]ed from blood [illegible] the juxtamedullary circulation and completely ex[illegible]ed from [illegible] tract[illegible] indicates the fraction of renal blood flow passing [illegible] Pilkington [illegible] 1965) found reduction of PAH-ex[illegible] and concluded that cortical fl[illegible] considerably [illegible] medullary flow did not change significantly. O[illegible] [illegible] 1949 and Houck (1951a) found no [illegible] change [illegible] PAH [illegible] adrenaline injection. Renal nerve stim[illegible] [illegible] of PAH or Diodrast in dogs [illegible] and

Shipley 1950, Houck 1951b Block, Wakim and Mann 1952b, Gömöri, Földi and Szabó 1961) suggesting a preponderance of cortical ischemia. It should be noted, however that the evidence for no extraction of PAH from juxtamedullary blood is entirely indirect (Pilkington *et al* 1965) and, in the view of the present author is insufficient to establish this as a method for evaluating blood flow distribution.

Aukland and Krog (1961) showed that single intravenous injections of adrenaline or noradrenaline invariably caused a transient rise in urine oxygen tension whereas the response to continuous infusions was variable. Although urine oxygen tension must partly be determined by medullary blood flow it was concluded that variations in medullary oxygen consumption may be of equal importance, and that urine oxygen tension cannot therefore be safely used as an index for medullary blood flow (Aukland 1962)

Based on external recording of renal Kr^{85} clearance Carriere *et al.* (1966) reported that adrenaline, noradrenaline and angiotensin selectively reduce cortical blood flow Although no details were given in their preliminary report, it follows from their method that they must have studied the effect of hour long infusions, and the results are therefore not directly comparable to those of the present study

The present experiments indicated that adrenaline, noradrenaline, angiotensin and renal nerve stimulation increase vascular resistance in both the cortical and the juxtamedullary circulation. but do not locate the vasoconstrictor effect to any special vascular segment It is generally accepted that in the cortex the main vascular resistance is confined to the pre- and post-glomerular arterioles. Since blood flow to the medulla has to pass through the juxtamedullary glomeruli with their afferent and efferent arterioles, it seems likely that the medullary circulation is also mainly governed by these arterioles, especially in view of the poorly-developed smooth muscle of the medullary vasa recta (von Möllendorff 1930) and the paucity of nerve fibres in this region (Mitchell 1951) Although some anatomical differences exist between cortical and juxtamedullary glomeruli (Selkurt 1963) it is therefore not surprising to find a common reaction pattern for the medullary and cortical circulation. The results are also well compatible with a parallel rise of cortical and juxtamedullary resistance during hemorrhagic hypotension in dogs (Aukland and Wolgast 1968) since all the factors studied here are mobilized in the defence reaction elicited by acute blood loss

I am greatly indebted to Civil Engineer S. Leraand for design and construction of the polarograph.

References

AUKLAND, K., Urine oxygen tension. Lack of correlation to some renal functions. *Acta physiol. scand.* 1962. *55*. 362—375.

AUKLAND, K., Study of renal circulation with inert gas measurements in tissue. *Proc. 3rd int. Congr. Nephrol. Washington 1966.* 1967a. *Vol. 1.* 188—200.

AUKLAND, K., Renal medullary heat clearance in the dog. *Circulat. Res.* 1967b. *20.* 194—203.

AUKLAND, K. and R. W. BERLINER, Renal medullary countercurrent system studied with hydrogen gas. *Circulat. Res.* 1964. *15.* 430—442.

Aukland K., B. F. Bower and R. W. Berliner, Measurement of local blood flow with hydrogen gas. *Circulat. Res.* 1964 *14* 164—187.

Aukland, K. and J. Krog, Influence of various factors on urine oxygen tension in the dog. *Act. physiol. scand.* 1961 *52* 350—365.

Aukland, K. and W. Wolgast. Effect of hemorrhage and retransfusion on intrarenal distribution of blood flow in dogs. *J. clin. Invest.* 1968. *47* I. press.

Berliner, R. W., N. G. Levinsky, D. G. D[illegible] and M. Eden. Dilution and concentration of the urine and the action of antidiuretic hormone. *Amer. J. Med.* 1958. *24* 730—744.

Block, M. A., K. G. Wakim and F. C. Mann. Circulation through the kidney during stimulation of the renal nerves. *Amer. J. Physiol.* 1952a. *169* 659—669.

Block, M. A., K. G. Wakim and F. C. Mann, Renal function during stimulation of renal nerves. *Amer. J. Physiol.* 1952b. *169* 670—677.

Bonsnes, R. W. and H. H. Taussky. On the colorimetric determination of creatinine by the Jaffé reaction. *J. biol. Chem.* 1945. *158* 581—591.

Carriere, S., G. D. Thorburn, C. C. C. O'Morchoe and A. C. Barger, Intrarenal distribution of blood flow during hemorrhagic hypotension. *Circulat. Res.* 1966. *19.* 167—179.

Christensen, G. C., Circulation of blood through the canine kidney. *Amer. J. [illegible]. Res.* 1952. *13* 236—245.

Daniel, P. M., C. N. Peabody and M. M. L. Prichard, Cortical ischemia of the kidney with maintained blood flow through the medulla. *Quart. J. [illegible] p. Physiol.* 1952. *37* 11—18.

Daniel, P. M., M. M. L. Prichard and J. N. Ward-McQuaid, An angiographic study of the effect of renin upon the renal circulation. *J. Physiol.* (Lond.) 1954. *124* 106—112.

Goodwin, W. E., R. D. Sloan and W. W. Scott. The "Trueta" renal vascular shunt" *J. Urol. (Baltimore)* 1949. *61* 1010—1027.

Gombos, P., M. Földi and G. Szabó, Renal function in the case of renal arterio-venous shunt formation. *Act. med. Acad. Sci. hung.* 1961. *17* 99—104.

Herd, J. A. and A. C. Barger, Simplified technique for chronic catheterization of blood vessels. *J. appl. Physiol.* 1964. *19* 791—792.

Hoyer, C. R., Alterations in renal hemodynamics and function during the intra[illegible]enous injection of epinephrine in the dog. *Amer. J. Physiol.* 1951a. *166* 649—657.

Hoyer, C. R., Alterations in renal hemodynamics and function in separate kidney during stimulation of renal arterial nerves in dogs. *Amer. J. Physiol.* 1951b. *167* 525—550.

Hughes-Jones, N. C., G. W. Pickering, P. H. Sanderson, H. Scarborough and J. Vandenbroucke, The nature of the action of renin and hypertensin on renal function in the rabbit. *J. Physiol.* (Lond.) 1949. *109* 288—307.

Kahn, J. R., L. T. Skeggs and N. P. Shumway. Studies of the renal circulation. *Circulatio* 1950. *1* 445—453.

Mitchell, G. A. G. The intrinsic renal nerves. *Act. anat.* (Basel) 1951. *13* 1—15.

Moyer, J. H., H. Conn, K. Markley and C. F. Schmidt. Attempt to demonstrate vascular by-pass in the kidney (The Trueta phenomenon). *Amer. J. Physiol.* 1950. *161* 250—258.

Von Möllendorff, W. *Handbuch der Mikroskopische Anatomie des Menschen.* Vol. 7 part 1. Berlin, Springer 1930. 129—131.

Ochwadt, B. and J. Schmidt, Über Temperatur und Kreislaufsmessungen in verschiedenen Abschnitte der Hundeniere. *Pflügers Arch. ges. Physiol.* 1954. *58* 261—274.

Pilkington, L. A., R. Binder, J. C. M. de Haas and R. F. Pitts. Intrarenal distribution of blood flow. *Amer. J. Physiol.* 1965. *208.* 1107—1113.

Reuben, F. C. and H. A. Schroeder, Can vascular shunting be induced in the kidney by [illegible] drugs? *J. clin. Inv. st.* 1949. *28* 114—123.

Sadler, H. H. and E. P. Tuttle, J. Thermodilution flow measurement in kidney tissue. *Circulatio.* 1963. *28* 795—796.

Schirmer, A. M. Focal blood flow measurements in cortex and medulla of kidney. *Amer. J. Physiol.* 1951. *167* 539—545.

S[illegible], E. E. The renal circulation. I. *Handbook of Physiology.* Sec. 2. Vol. II. Circulation. Ed. by Hamilton W. F. and P. Dow. Washington D.C. Amer. Physiol. Soc. 1963. 1457—1516.

Smith, H. W., N. Finkelstein, L. Aliminosa, B. Crawford and M. Graber, The renal clearance of substituted hippuric acid derivatives and other aromatic acids in dog and man. *J. [illegible] I[illegible] st.* 194[illegible] *24* 388—404.

Study, R. and R. F. Shipley. Comparison of direct with indirect renal blood flow extraction of [illegible] lin and diodrast, before and during acute renal nerve stimulation. *Amer. J. Physiol.* 1950. *163* 44[illegible]—453.

T[illegible], K. and P. Dieter. Die Diurese bei arteriellen Drucksteigerung. *Pflügers Arch. ges. Physiol.* 1962. *274* 567—580.

T[illegible] J., A. E. Barclay, P. M. Daniel, K. J. F[illegible] and M. M. L. Prichard [illegible] *of the renal circulation.* Springfield, Illinois, Charles C. Thomas, 1947.

Acta physiol. scand. 1968, 72, 510—517

From the Institute of Anatomy and Histology and Department of Obstetrics and Gynecology at Malmö, University of Lund, Sweden

Considerations on the Cause of Disappearance of the Adrenergic Transmitter in Uterine Nerves during Pregnancy

By

NILS-OTTO SJÖBERG

Received 28 August 1967

Abstract

SJÖBERG, N.-O. *Considerations on the cause of disappearance of the adrenergic transmitter in uterine nerves during pregnancy.* Acta physiol. scand. 1968. 72. 510—517.

With combination of fluorescence histochemical and fluorimetric techniques has been demonstrated that the smooth muscle coats of the guinea-pig uterus has fairly rich supply of "short" adrenergic neurons, i.e. neurons arising in the vicinity of the organ, emitting a green fluorescence owing to their noradrenaline content. During the last period of pregnancy the neuronal fluorescence as well as the uterine noradrenaline disappears. Using animals with unilateral pregnancy it has been possible to establish that despite marked difference in weight and distention between the two horns there is no difference in the reaction of the adrenergic nerves during the period of pregnancy studied (3?—63 days of gestation). Hence, it seems probable that the marked changes in transmitter content found during the latter part of pregnancy are due to humoral rather than to mechanical factors.

Recent investigations with combined histochemical and chemical methods have demonstrated that the neuronal noradrenaline content of the oviduct, uterus, and vagina in the rabbit increases markedly during the former half of pregnancy (Rosengren and Sjöberg 1967 a). In contrast, ovarian noradrenaline remains unchanged. In the above-mentioned investigation this increase in transmitter content in the uterus and vagina was found to be followed by a reduction in the adrenergic transmitter content later on during pregnancy. The reduction was most prominent in the uterus, where barely any noradrenaline-containing nerves could be demonstrated towards the end of pregnancy.

It has previously been established in denervation experiments (*Owman et al.* 1966) that the uterus and vagina of the rabbit are innervated by "short" adrenergic neurons, i.e. neurons arising in the vicinity of these organs, while the ovary receives its adrenergic innervation by way of ordinary "long" adrenergic neurons from the sympathetic chain. The oviduct was found to be inner[illegible] th types of neur[illegible]

As one possible explanation of the above mentioned disappearance of adrenergic transmitter from the pregnant uterus and from the vagina it was suggested that it might simply be the result of a mechanical interference with the neurons during the pronounced hyperthophy of the muscle coat and distension in these two organs.

It seemed warranted to test this possibility by comparing the transmitter content of the uterine horns in animals with litters in only one of the horns, i.e. with pronounced hypertrophy of the muscle coat and distention on one side only. In order to obviate the otherwise necessary surgical operation, the guinea pig was chosen for the experiments, because in this species pregnancy is often unilateral.

Material and methods

All together 27 pregnant (32nd—63rd day of gestation) and 37 non-pregnant female guinea-pigs were used. Six of the pregnant animals had litters in one uterine horn only. The normal length of gestation in this species is about 63 days.

Using the histochemical fluorescence technique of Falck and Hillarp (Falck 1962, Falck et al. 1962, Corrodi and Hillarp 1963, 1964) neuronal noradrenaline was demonstrated in freeze-dried uterine preparations treated with formaldehyde gas at +80° C for 1 hr according to Falck and Owman (1965). The noradrenaline content was determined fluorimetrically by the method of Bertler et al. (1958) as modified by Häggendal (1963). In the pregnant animals the total noradrenaline content was determined separately in the two uterine horns including their cervical portions. From each horn minute pieces were alway taken for histochemical analysis. In animals where the uterine horns were not used for chemical determinations several larger preparations wer investigated histochemically.

In 3 non-pregnant animals the vascular system was demonstrated by India ink injected in the abdominal aorta (300 mm Hg for 3 min., see further Rosengren and Sjöberg, 1967 b) in order to confirm the presence of non-vascular adrenergic nerves.

Results

The muscular wall of the guinea pig uterus consists of an outer longitudinal and an inner circular smooth muscle layer separated by a well developed vascular plexus (Fig. 1). *In the non-pregnant guinea pig* a large number of these vessels were enclosed by plexuses of varicose nerves (Fig. 2) emitting an intense green fluorescence under the conditions used. These plexuses were characteristically superimposed upon the media of the vessel wall. By India ink injection the vascular innervation could clearly be distinguished from a moderate number of non-vascular fluorescent varicose terminals. Such nerves (Fig. 1 and 2) were present both in the inner circular and in the outer longitudinal, smooth muscle layers, usually running parallel to, and contiguous with the muscular fibres. However when accompanying the vessels some nerves in the circular coat had a more radial direction. The endometrium showed few scattered fluorescent nerve terminals close to vessels adrenergic nerves were never seen to reach the epithelium. Thick bundles consisting both of smooth preterminal and of some varicose terminal axons ran longitudinally both in the perimetrium and between the two myometrial layers. The fluorescence intensity of the preterminals was considerably lower than in the varicose terminals.

In order to ascertain whether the adrenergic nerves reach the smooth muscle of the guinea pig uterus through the hypogastric nerves or — as in the rabbit — from peripheral relays the inferior mesenteric ganglia were extirpated together with 3

Acta physiol. scand. 1968. 72. 510—517

From the Institute of Anatomy and Histology and Department of Obstetrics and Gynecology at Malmö, University of Lund, Sweden

Considerations on the Cause of Disappearance of the Adrenergic Transmitter in Uterine Nerves during Pregnancy

By

Nils-Otto Sjöberg

Received 28 August 1967

Abstract

Sjöberg, N.-O. *Considerations on the cause of disappearance of the adrenergic transmitter in uterine nerves during pregnancy.* Acta physiol. scand. 1968. 72. 510—517.

With combination of fluorescence histochemical and fluorimetric techniques it has been demonstrated that the smooth muscle coats of the guinea-pig uterus have a fairly rich supply of adrenergic neurons, neurons arising in the vicinity of the organ, emitting green fluorescence owing to their noradrenaline content. During the last period of pregnancy the neuronal fluorescence as well as the uterine noradrenaline disappears. Using animals with unilateral pregnancy it has been possible to establish that despite a marked difference in weight and distention between the two horns there is no difference in the reaction of the adrenergic nerves during the period of pregnancy studied (32—63 days of gestation). Hence, it seems probable that the marked changes in transmitter content found during the latter part of pregnancy are due to humoral rather than to mechanical factors.

Recent investigations with combined histochemical and chemical methods have demonstrated that the neuronal noradrenaline content of the oviduct, uterus, and vagina in the rabbit increases markedly during the former half of pregnancy (Rosengren and Sjöberg 1967 a). In contrast, ovarian noradrenaline remains unchanged. In the above mentioned investigation this increase in transmitter content in the uterus and vagina was found to be followed by a reduction in the adrenergic transmitter content later on during pregnancy. The reduction was most prominent in the uterus, where barely any noradrenaline-containing nerves could be demonstrated towards the end of pregnancy.

It has previously been established in denervation experiments (Owman *et al.* 1966) that the uterus and vagina of the rabbit are innervated by "short" adrenergic neurons, i.e. neurons arising in the vicinity of these organs, while the ovary receives its adrenergic innervation by way of ordinary "long" adrenergic neurons from the sympathetic chain. The oviduct was found to be innervated by both types of neurons.

Table I. Noradrenaline content and concentration in guinea-pig uterus after removal of the hypogastric nerves together with the inferior mesenteric ganglia

Animals	µg/pair Mean ± S.E.( )	µg/g Mean ± S.E( )	Weight g/pair Mean ± S.E.(
Controls	0.52 ± 0.04 (13)	0.75 ± 0.08 (13)	0.80 ± 0.11 (13
Denervated	0.42 0.02 (7)	0.73 ± 0.06 (7)	0.58 ± 0.03 (7)

Difference between non-operated and denervated animals not significant (P > 0.05)

of the hypogastric nerves of 10 animals. After 7 day the animals were killed and both uterine horns including their cervical portions were dissected out for fluorescence microscopy and chemical determination of noradrenaline. Fluorescence microscopy showed that the operation did not interfere with the arrangement of adrenergic nerves the *noradrenaline content* in the uterus remained unchanged (*Table I*). Besides the uterine horns, also the agina was dissected out for histochemical analysis. In greement with previous findings in the rabbit (Owman and Sjöberg 1966) cat (Rosengren and Sjöberg 1967 b) and man (Owman *et al.* 1967) clusters of fluorescent nerve cells (Fig. 3) were found scattered in the periphery of the upper portion of the aginal wall. The results strongly indicate the presence of a peripheral elay

During the period of the gestation tudied — 32 to 63 day after mating — the number of fluorescent nerves (*i.e.* nerves isible because of their formaldehyde-induced fluorescence) in the uterus was markedly decreased in all the animals. Fluorescence microscopic analysis of a large number of preparations often failed to demonstrate any fluorescent nerv in the smooth muscles of any part of the uterine horns (Fig. 4) Occasionally isolated aricose terminals were found in relation to blood essels. The microscopic findings were the same irrespective of the presence or absence of fetuses in the uterine horns (Fig 5) A somewhat larger number of fluorescent nerves persisted in the cervical region and there too, they seemed to be



Fig. 3. The figure illustrates cluster of fluorescen adrene ic ganglion cells present in the pe pher of the aginal wall Modera r ic fluorescence. Below the al groups of chromaffin onspicuous fluorescence ent 200 ×



Fig. 4. Uterine horn. 60 days of pregnancy. The musculature and vessels are completely devoid of fluorescent noradrenaline-containing nerves. 200 ×

Fig. 5. Uterine horn containing no litters. 63 days of pregnancy. No fluorescent nerves are demonstrable in the musculature or around the vessels. Some of the vascular walls has conspicuous auto-fluorescence. 310 ×

Fig. 6. Cervical region. 63 days of pregnancy. Some fluorescent nerves are found, essentially in relation to vessels. 125 ×

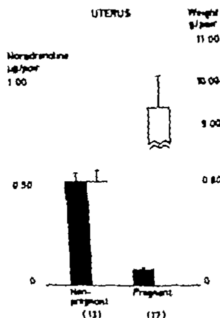


Fig. 7. Total noradrenaline content and weight (expressed me — S.E.M.) of uterus in non-pregnan s and animals pregnant for 32—63 da ber of determinations in brackets. F lle ue noradrenaline content. Unfilled ol ean weight.

essentially l. Fig. 6). The findings are in close agreement with the fluorimetric det a which showed a highly significant decrease in uterine noradrenaline t (Fig. 7). The difference in the total amount of per

TABLE II. Noradrenaline content in pregnant uterine horn containing or lacking fetuses

Horn of pregnant uterus	µg/horn Mean ± S.E. ( )	Weight (g) Mean ± S.E. (n)
Containing fetuses	0.04 ± 0.02 (6)	6.81 ± 1.22 (6)
Lacking fetuses	0.07 ± 0.02 (6)	1.04 ± 0.17 (6)

noradrenaline between uterine horns with or without fetuses during pregnancy (Table II) was no significantly different ($P > 0.05$). The uterine horn containing no fetuses was relatively small and weighed only about twice that of a horn from a non-pregnant animal. On the other hand, the uterine horn containing fetuses was markedly enlarged and distended, weighing more than six times that of the horn without fetuses (Table II).

Discussion

The present investigation demonstrates that the uterus of the non-pregnant guinea pig has a rich supply of adrenergic nerves distributed in the smooth muscle coats and around vessels. Results of denervation experiments in this and previous (Owman et al. 1966, Rosengren and Sjöberg 1967 b) studies make it highly probable the adrenergic nerves to the uterine musculature are of the "short" adrenergic neuron type, arising in ganglia at the utero-vaginal junction. The arrangement of the adrenergic nervous system in the uterus thus resembles that in the rabbit (Owman and Sjöberg 1966, Owman et al. 1966) and cat (Rosengren and Sjöberg 1967 b).

During the latter part of pregnancy there was in the uterus a marked decrease in the number of nerves exhibiting a formaldehyde induced fluorescence characteristic of a primary catecholamine. Only very few fluorescent nerve fibres — most if not all of them being vascular — persisted. This was particularly evident in the cervical region. Comparison of the two uterine horns in unilaterally pregnant animals revealed that this conspicuous change in the fluorescent uterine adrenergic nerves occurred whether the uterine horn contained fetuses or not. The histochemical findings corresponded well with the pronounced fall in uterine noradrenaline registered fluorimetrically.

The uterine horn containing fetuses is distended and much larger than the contralateral horn without fetuses, but the noradrenaline content shows an equally pronounced reduction in both uterine horns during pregnancy. Therefore it is highly improbable that a simple mechanical factor should be responsible for the disappearance of adrenergic transmitter in the uterine nerves during pregnancy (Rosengren and Sjöberg 1967 a). The findings rather favour the possibility that the neurons have been affected by a yet unknown, probably humoral factor.

In contrast to the fluorescent muscular adrenergic nerves, some of the fluorescent

ves related to vessels persisted. It should be recalled that the vascular adrenergic nerves are of the "long" type whereas the smooth muscles are innervated by "short" adrenergic neurons. This might indicate that the two types of neurons are not affected in the same way by the assumed humoral factor.

The investigation was supported by grants from the Association for the Aid of Crippled Children, New York, the Swedish Medical Research Council (projects No. B67-12X-712-02A and B67-14X-56-03A) and the Medical Faculty at the University of Lund.

References

Bertler, A., A. Carlsson, E. Rosengren and B. Waldeck, A method for the fluorimetric determination of adrenaline, noradrenaline and dopamine in tissues. Kungl. Fysiogr. Sällsk. Lund. Förh. 1958. 28 121—123.

Corrodi, H. and N. A. Hillarp, Fluorescenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 1. Identifizierung der fluoreszierenden Produkte aus Modellversuchen mit 6, 7-Dimethoxyisochinolinderivaten und Formaldehyde. H lv kim. Acta 1963. 46 2425—2430.

Corrodi, H. and N. A. Hillarp, Fluorescenzmethoden zur histochemischen Sichtbarmachung von Monoaminen. 2. Identifizierung des fluoreszierenden Produktes aus Dopamin und Formaldehyd. H lv kim. Acta. 1964. 47 911—918.

Falck, B., Observations on the possibilities of the cellular localization of monoamines by fluorescence method. A ta physiol. scand. 1962. 56. Suppl 197 1—25.

Falck, B., N.-A. Hillarp, G. Thieme and A. Torp, Fluorescence of catechol amines and related compounds condensed with formaldehyde. J. Histochem. Cytochem. 1962. 10 348—354.

Falck, B. and CH. Owman, A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. Act Univ Lund. II 1965. 7 1—23.

Häggendal, J. An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues. A ta physiol. scand. 1963. 59 42—254.

Owman, CH., E. Rosengren and N.-O. Sjöberg, Origin of the adrenergic innervation to the female genital tract of the rabbit. Lif S i 1966. 5 1389—1396.

Owman, CH., E. Rosengren, and N.-O. Sjöberg, Histochemical and chemical investigation on the adrenergic innervation of the human female reproductive organs. Obstet. Gynec. 1967. In press.

Owman, CH. and N.-O. Sjöberg, Adrenergic nerves in the female genital tract of the rabbit. With remarks on cholinesterase-containing structures. Z. Zellforsch. 1966. 74 182—197.

Rosengren, E. and N.-O. Sjöberg, Changes in the amount of adrenergic transmitter in female genital tract of rabbit during pregnancy. Act physiol. scand. 1968. 72 412—424.

Rosengren, E. and N.-O. Sjöberg, The adrenergic nerve supply to the female reproductive tract of the cat. Amer. J. Anat. 1967 b. 121 271—284.

Acta physiol. scand. 1968. 72. 518—532

From the Department of Physiology, University of Göteborg, Sweden

Effects of Carotid Baroreceptor Reflexes on Venous Tone in Skeletal Muscle and Intestine of the Cat

By

John Hadjiminas[1] and Bengt Öberg

Received 8 September 1967

Abstract

Hadjiminas, J. and B. Öberg. *Effects of carotid baroreceptor reflexes on venous tone in skeletal muscle and intestine of the cat.* Acta physiol. scand. 1968. 72. 518—532.

The engagement of skeletal muscle and intestinal veins in baroreceptor reflexes was quantitatively evaluated by comparing the reflex responses of the resistance and capacitance vessels with those obtained with direct stimulation of the regional vasoconstrictor fibres. Such comparisons revealed that the reflex constriction of skeletal muscle veins, following diminution ... ptor activity was, in general, considerably smaller than the concomitant resistance constriction. This discrepancy points to a definitely lower reflex discharge rate in the ... constrictor fibres. When, however, vasomotor centre excitation, following withdrawal of ... receptor restraint, was intense, the ensuing venoconstriction in skeletal muscles became as pronounced as that of the resistance vessels. In the intestine the reflex responses of the capacitance and resistance vessels were always equally pronounced, indicating an essentially uniform reflex discharge rate in all intestinal vasomotor fibres. However, in comparison with the muscle resistance vessels, the intestinal vascular bed seems to be only moderately involved in baroreceptor reflexes. It is concluded that the capacitance vessels in the studied circuits participate in baroreceptor reflexes but less extensively than the muscle resistance vessels. The functional significance of the differentiated reflex vascular response pattern in skeletal muscles is discussed with special reference to the consequences for the net fluid exchange across the capillaries.

Peripheral vascular adjustments in arterial baroreceptor reflexes have been widely studied (e.g. Heymans and Neil 1958, Löfving 1961, Poloxa and Rossi 1961, Öberg 1964). It is thus well established that an unloading of the baroreceptors produces a reflex constriction of the resistance and capacitance vessels in many circuits and also a net capillary absorption of tissue fluid, mainly from skeletal muscles (Öberg 1964). Few attempts have, however, been made to evaluate to what extent the responses of the various individual parallel-coupled beds with their functionally different, series-coupled sections contribute to the overall cardiovascular adjustments, produced e.g. by a diminution of baroreceptor activity. In this respect more in-

[1] On WHO stipend. present address: Department of Physiology, University of Athens, Goudi, Athens (609) — Greece.

formation is required for a full understanding of the control of circulatory homeostasis. Some relevant data exist, however. For example the resistance vessels in the various circuits do not seem to be uniformly engaged in baroreceptor reflexes, but those in the skeletal muscles are usually more strongly activated than the resistance vessels of the skin, kidney and probably also of the intestine (Opitz and Smyth 1937, McGiff and Aviado 1961, Löfving 1961, Öberg 1964). This suggests that, as far as flow resistance changes in baroreceptor reflex patterns are concerned, they can often be mainly ascribed to shifts in skeletal muscle vessel tone. The background of this differentiated engagement of the various vascular beds seems to be, at least in part, that the neuron pools in the bulbar vasomotor centre, conveying their efferent discharge to the various circuits become activated to a different extent when they are gradually released from the baroreceptor inhibitory influence (Folkow, Johansson and Löfving 1961).

Whether a similar differentiation exists concerning the reflex control of the resistance and capacitance vessels in one and the same circuit or with regard to the control of the capacitance vessels in the different vascular beds, as is the case with the resistance vessels, is less well known. Considering the importance of the capacitance section of the cardiovascular system for the maintenance and regulation of venous return and cardiac output one might expect that the veins should be strongly involved in various homeostatic reflex mechanisms. This does not seem to be generally the case, however. Preliminary experiments in this laboratory (see Folkow 1962) suggested that the capacitance section of a skeletal muscle bed was less engaged than the corresponding resistance vessels when the carotid arteries were occluded. In a recent study utilizing a different technique, Browse, Donald and Shepherd (1966) have also claimed that the veins of the limbs are far less engaged than the arterioles in baroreceptor reflex mechanisms.

The present study was undertaken to explore to what extent the precapillary and postcapillary vascular sections of the skeletal muscles and the intestinal vascular beds are reflexly activated during graded levels of carotid sinus hypotension. Repeated comparisons of the magnitude of the resistance and capacitance responses were made when, on one hand, the vasomotor fibres were reflexly activated by inducing carotid sinus hypotension and when, on the other, all, or the majority, of the regional vasomotor fibres were directly activated by means of well defined electrical stimulations.

Methods

Experiments [illegible] performed on 40 cats, anesthetized with chloralose, 30—50 mg/kg body weight. I[illegible] animals the vascular sections in skeletal muscle were studied, utilizing the skinned hindquarters, which were prepared according to the technique described by Mellander (1960). The pa[illegible] should be consulted for details. In principle the hindquarters were isolated from the res[illegible]e animal except for the aorta, the inferior caval vein and the sympathetic trunks. The [illegible]nd the pa[illegible] were excluded from the preparation by tight ligatures and the skin [illegible] freed from underlying tissue. After these procedures the hindquarters consists of [illegible] per cent skeletal muscle and 33 per cent skeleton. — The hindquarters were plac[illegible] [illegible] refilled temperature-controlled plethysmograph, connected to [illegible] piston recorder fo[illegible]ements of tissue volume changes. Rapid, phasic volume changes can, [illegible]

this technique, be ascribed to shifts in regional blood content, while slower continuous alterations of volume reflect transcapillary fluid movements (Mellander 1960). — After heparine (5 mg/kg) the inferior caval vein was cannulated and the venous effluent was diverted through a Gaddum recorder for blood flow measurements. The blood was returned to the animal via a funnel connected to the central end of the inferior caval vein. The flow recorder could be placed at varying heights above the preparation and the venous outflow pressure could be accordingly changed and set at any desired level. — For direct activation of all, or at least the great majority of the vasoconstrictor fibres to the hindquarters, bipolar electrodes were placed on the intact sympathetic trunks at that level between L1 and L3 where maximal stimulation effects were obtained. Stimulations were delivered by a Grass stimulator at frequencies between 1 to 10 imp/sec and with a duration and voltage that produced maximal responses for a given frequency to ensure activation of all fibres placed at the electrode. Atropine, 0.3—0.5 mg/kg was given to avoid any interference from the cholinergic vasodilator fibres at direct stimulation.

In 5 expts., intestinal vascular reactions were studied. For this purpose a suitable length of small intestine was isolated and prepared according to Folkow, Lundgren and Wallentin (1963). After cannulation of the mesenteric vein for blood flow measurements with a drop chamber the intestine was placed in a plethysmograph filled with Tyrode solution, kept at 37° C and connected to a piston recorder for volume measurements. — The vasoconstrictor fibres passing along the mesenteric artery were cautiously dissected free but otherwise left intact, and placed on a fine bipolar silver electrode for direct stimulation. The duration and voltage of the stimuli were set to produce maximal responses at a given frequency. — Atropine, 0.3—0.5 mg/kg, was given to block the cholinergic fibres to the intestinal smooth muscle and glands to avoid interference from intestinal motility and secretion on the recorded vasomotor reactions at direct stimulation of the nerves.

Carotid sinus baroreceptor reflexes were generally elicited simply by bilateral occlusion of the common carotid arteries, but in some experiments a modified Moissejeff preparation was used. The vagal nerves were in most cases cut in the neck in the course of the experiment. The arterial blood pressure was recorded from the inferior mesenteric artery. The arterial inflow pressure to the hindquarters and the intestine could be kept constant during the various experimental procedures by adjustments of a screw-clamp, placed around the abdominal aorta proximal to the inferior and superior mesenteric arteries, expertly or by connecting the arterial side of the circulation to a simple pressure compensator. — At the beginning of the expt. the venous outflow pressure in the tissue studied was set at 10—15 mm Hg. Passive mobilization from the veins, secondary to the fall in venous pressure when precapillary vessels constrict in the course of vasoconstrictor fibre activation, will then be rather small. The recorded changes in regional blood volume can therefore be ascribed mainly to active venoconstriction (Öberg 1967). With the mentioned venous outflow pressure levels an isovolumetric state was practically obtained, i.e. the capillary pressure attained such a value that capillary filtration balanced capillary absorption almost exactly.

Results

Fig. 1 shows recordings from one experiment, where the reactions of a skeletal muscle vascular bed (hindquarter preparation) to bilateral carotid occlusion and direct stimulation of the regional vasoconstrictor fibres, respectively, were analysed. The venous outflow pressure was initially set at 10 mm Hg and an isovolumetric state was achieved. The arterial inflow pressure was kept essentially constant throughout by adjustments of the aortic screw-clamp. Three periods of bilateral carotid occlusion are shown. This procedure can be seen to induce a constriction of the resistance vessels, increasing peripheral resistance (PRU) 60, 55 and 90 per cent, respectively. There is also a simultaneous reduction of the blood content of the hindquarters, as revealed by the fast initial phase of vol ume reduction, implying a constriction of the capacitance vessels. Furthermore a net transcapillary fluid absorption ensues as indicated by the slow, continuous volume decrease, proceeding as

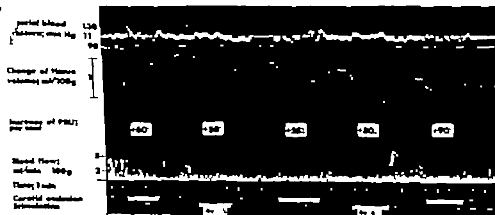


Fig. 1 Cat 3.2 kg. Chloralose 35 mg/kg. Atropine 0.5 mg/kg. Effects of bilateral carotid occlusion and regional vasoconstrictor fibre stimulation on blood flow and tissue volume of the hindquarters. The capacitance vessel responses are reflected in the rapid, phasic volume changes. — *Note the small capacitance vessel responses to carotid occlusion as compared with those obtained at vasomotor fibre stimulation, despite essentially identical resistance vessel responses (increases of PRU) in the two cases.*

long as the carotid arteries are clamped. The blood expulsion produced by the constriction of the capacitance vessels during carotid occlusion amounts to 0.2—0.3 ml/100 g tissue. This corresponds to approximately 10 per cent of the regional blood content, assumed to amount to approximately 2.5—3.0 ml/100 g muscle (Mellander 1960). Direct, electrical stimulation of the regional vasomotor fibres with supramaximal strength at a frequency of 3 and 4 imp/sec respectively can be seen to produce vascular adjustments, *qualitatively similar to those obtained at bilateral* carotid occlusion. From a *quantitative* point of view however the vascular responses differ in the two cases. Thus, while the resistance vessel responses are comparable in magnitude in the two cases, a direct stimulation of the vasoconstrictor fibres can be seen to produce a considerably larger reduction in blood content, comprising ... 0.45 ml and 0.6 ml/100 g tissue, respectively or about 15 to 20 per cent of the regional blood content.

The mentioned *quantitative* differences in the vascular responses when the regional vasomotor fibres were activated by direct electrical stimulation, on one hand and reflexly activated by partial unloading of the baroreceptors, on the other were present in all the experiments with the skeletal muscle preparation, ... the reflex sympathetic activation was slight or moderate ... experiments where the reflex activation was so intense as to ... decrease in flow resistance which sometimes occurred upon ... in addition the vagal nerves were cut and the ... slightly ... the venous responses became quantitatively similar to those ... by direct stimulation. This phenomenon is illustrated ... of reflex vasoconstrictor fibre activation in ... hindquarter ... were here produ...

clamping one carotid artery (panel A) both carotid arteries (panel B) a) clamping both carotid arteries while the animal was slightly hypoventilated (panel C) and the induced vascular responses compared with those obtained at stimulation of the regional vasoconstrictor fibres with different frequencies. When the reflex vasoconstrictor activation was low (one carotid artery clamped) or moderate (both carotids clamped) the resistance vessel responses can be seen to approximately equal those obtained at direct stimulation with 2.5 and 5 imp sec, respectively while at the same time the *reflexly* induced blood mobilizations are considerably smaller than those obtained at direct stimulation. This is thus in accordance with the findings presented in Fig 1 When, however the reflex activation of the vasomotor fibres become intense so as to increase flow resistance some 5—6 times (panel 3) the reflexly produced blood mobilization becomes equally pronounced as that obtained at direct stimulation. This suggests that as long as the baroreceptors maintain the vasomotor centre activity at a low or moderate level the extent of venoconstriction is small or perhaps even insignificant in the skeletal muscles, as compared with the extent of engagement of precapillary resistance vessels. However with a further diminution of baroreceptor inhibitory influence on the asomotor centre, the eins become more and more involved so that ultimately both pre- and postcapillary vascular sections are activated to the same, supposedly maximal, extent.

It seems at present most reasonable to assume that the mentioned unequal engagement of the skeletal muscle resistance and capacitance vessels in baroreceptor is related to differences in the a erage impulse discharge rate of the aso- ctor fibres running to pre and postcapillary vascular sections, respecti ely liscussion) This reflexly produced average firing rate in the respective sets of constrictor fibres can then be approximately evaluated by comparing the reflex vascular responses with those obtained at direct electrical stimulation of the asoconstrictor nerves with known frequencies. Such evaluations can be made n Fig 3 which show collected data from 20 expts. with the hindquarter preparation of the type illustrated n Fig 1 and 2 The resistance vessel responses (per cent increase of flow resistance) are here plotted against the simultaneously recorded enous responses (ml blood mobilized) obtained both with direct timulation of the vasoconstrictor fibres (open circles) and reflexly with graded unloadings of the carotid sinus baroreceptors (dots) The impulse frequency of the directly applied *stimulations* are given, and the range of riation of the vascular responses in the indi idual experiments to a given stimulation frequency indicated by the shaded areas on the "*frequency*" axis. *It can be seen that with direct* stimulation of the regional asoconstrictor fibres, maximal enous esponses (1 to 1 1 ml blood mobilization 100 g tissue, which comprises roughly 35 to 40 per cent of the total regional blood content) are obtained with stimulat on frequencies of 6, sometimes 8 imp sec while maximal resistance essel responses appro mately 500 per cent increase of flow resistance) are reached first with stimulation frequencies of 12 to 15 imp sec in accordance with the findings by Mellander 1960 Fig. 3 makes it further evident

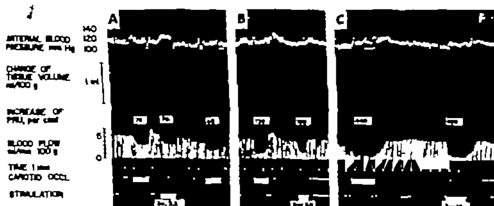


Fig. 2. Cat 2.8 kg. Chloralose 40 mg/kg. Atropine 0.4 mg/kg. Both vagal nerves cut in the neck. — Vascular responses in the hindquarters produced by direct vasomotor fibre stimulation with varying frequencies and by occlusion of the right carotid artery (panel A) by occlusion of both carotid arteries (panel B) and by occlusion of both carotid arteries while the animal was slightly hypoventilated (panel C)

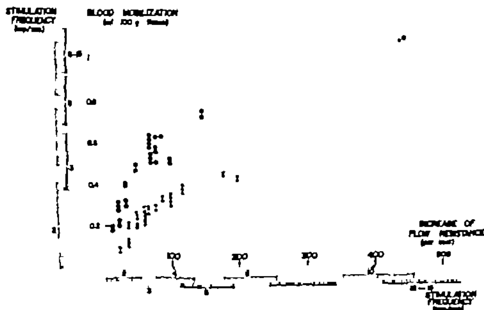


Fig. 3. Correlation between capacitance vessel responses (expressed in terms of blood mobilization) and resistance vessel responses (per cent increase of PRU) in the skinned hindquarters, produced by unloading the baro-receptors (dots) and by stimulation of the regional vasoconstrictor fibres (open circles). — The frequencies used at direct stimulation are shown and the ranges of variation of the vascular responses in the various experiments at given stimulation frequencies are indicated by the shaded areas at the frequency axes. The data have been collected from 20 experiments.

that the reflex vascular responses differ from those obtained at direct stimulation demonstrated in Fig 1 and 2. Thus, for any given increase of PRU the *ref* venous responses are considerably smaller than those induced by *direct* stimulation as long as the rise of PRU is moderate. When the PRU increase becomes very pronounced, however the corresponding reflex responses of the capacitance vessels also becomes very marked and equal those obtained with direct stimulation.

If the actually recorded reflex changes of vascular tone are translated into reflex shifts in the average impulse discharge rate of the vasoconstrictor fibres, it can be seen that a reflex rise of PRU by 100 per cent which implies a firing rate of approximately 4 imp sec in the fibres running to the precapillary resistance vessels, is accompanied by a blood mobilization of roughly 0.3 ml 100 g which corresponds to a discharge rate of only 2 imp sec in the fibres running to the postcapillary capacitance vessels. Similarly when PRU is reflexly increased by 200 per cent, implying 6 imp sec in precapillary vasomotor fibres, the simultaneous capacitance response corresponds to only 3 imp sec in postcapillary vasomotor fibres. When a firing rate of 8 imp sec is reflexly produced in precapillary" constrictor fibres (PRU increase of roughly 300 per cent) the corresponding impulse frequency in postcapillary vasomotor fibres amounts to only 4 imp sec etc. Maximal reflex resistance vessel responses can be seen to imply a firing rate of at least 10—12 imp sec in the corresponding constrictor fibres. The venous responses are at the same time also maximal which, however might very well be equivalent to an impulse rate of only some 6 imp sec in the corresponding vasoconstrictor fibres, although still higher uencies cannot be ruled out. However there is throughout a tendency towards 1 ratio with respect to the reflexly produced firing rates in precapillary postcapillary vasoconstrictor fibres, respectively in a skeletal muscle vascular when the baroreceptor inhibitory influence on the vasomotor centre is gradually diminished.

It can be argued that the stimulation frequencies given in the preceding figures may not be entirel correct, since a certain summation of the spontaneous, tonic impulse discharge of the vasoconstrictor fibres and the artificially applied electrical stimulations may ha ccurred To test this, and also to estimate approximately the firing rate of this resting tonic activity of the constrictor fibres, running to the pre- and postcapillary vascular sections, respectively the hindquarter preparation was denervated at the end of the experiment in 3 animals, and complete stimulation frequency response curves estimated It was then found that with impulse frequencies above 2 imp sec the PRU increased for any given stimulation frequency to approximately the same valu irrespecti whether the preparation was innervated or denervated, the differences being within the range of variation given in Fig 3. Therefore with stimulation frequencies above 2 imp sec a significant summation of impulses did not seem to occur nd the stimulation frequencies given in the figures can consequently be considered to be essentially correct. However at stimulation frequencies lower than 2 imp sec th PRU increase was unequivocally somewhat larger in the innervated preparation indicating that a certain summation occurred

in this stimulation range. — The experiments on the denervated preparation further showed, that a stimulation frequency of 1 2—1 imp sec was necessary to increase flow resistance to the pre-denervated level, indicating a "resting" discharge rate of this order in the fibres running to the precapillary resistance vessels. This value agrees with the figure given by Lundgren *et al.* (1964) However a frequency less than 0.5 imp/sec was usually sufficient to reduce the regional blood content in the denervated preparation to the pre-denervated value, indicating a definitely lower spontaneous firing rate in the vasoconstrictor fibres running to the postcapillary vessels. Thus, the mentioned 2 to 1 ratio between the discharge rates in pre and postcapillary constrictor fibres seems to prevail also with respect to the resting spontaneous vasoconstrictor fibre activity

As contrasted to the case in skeletal muscle, the *intestinal* vascular responses were found to be essentially identical either the vasoconstrictor fibres were activated reflexly by unloading the carotid sinus baroreceptors or they were directly stimulated with well defined electrical stimuli. There was thus no clearcut difference with regard to the extent of venoconstriction in the two cases. Fig 4 illustrates recordings from one representative experiment in this series. It shows that a given increase of flow resistance is accompanied by approximately the same magnitude of blood mobilization irrespective whether the constrictor fibres were stimulated directly or activated reflexly — In Fig 5 data from 4 expts. on the intestinal preparation have been collected. The resistance vessel responses, obtained at direct stimulation (open circles) and reflexly *via* the baroreceptors (dots) are here plotted against the stimul

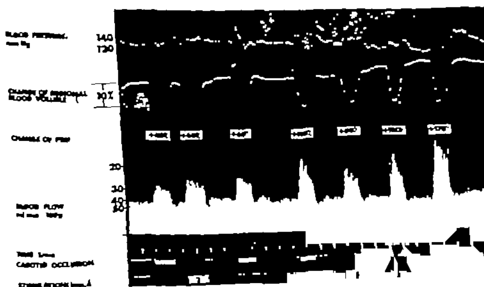


Fig. 4 Cat 3.1 kg. Chloralose 40 mg/kg. Atropine 0.5 mg/kg. The ... to the neck. Effects of bilateral carotid occlusion and regional vasoconstrictor fibre stimulation on blood flow and tissue volume in the intestine.

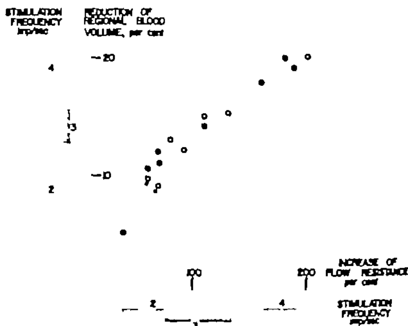


Fig. 5 Correlation between capacitance and resistance vessel responses in the intestine, produced by carotid occlusions (dots) and direct regional vasoconstrictor fibre stimulation (open circles) — The data has been collected from 4 experiments.

taneously recorded venous responses. It can be seen that the *reflex* vascular adjustments agree well with those obtained at direct stimulation also from a quantitative point of view. This should then imply that the reflexly induced impulse discharge rate was essentially similar in fibres running to pre- and postcapillary vascular sections, respectively, in the intestine in contrast to the case in skeletal muscle. It can also be seen in Fig. 5 that the firing rate of the intestinal vasoconstrictor fibres were comparatively low even when all baroreceptor inhibitory influence on the vasomotor centre was supposedly eliminated and did never seem to exceed 4 imp/sec in this series of experiments.

The intestinal blood flow responses to a prolonged vasoconstrictor fibre activation usually showed an *initial* more marked flow reduction, followed by a gradual increase of blood flow towards resting levels despite a continuous stimulation. This phenomenon has been called autoregulatory escape of the intestinal resistance vessel constriction from the vasomotor fibre influence (Folkow *et al.* 1964). The autoregulatory escape seems to be most expressed when the vasomotor fibre excitation is very pronounced, which implies that the overall flow resistance in the steady state condition, is often low in the intestine when the constrictor fibres are strongly activated. For such reasons, both resistance and capacitance vessel responses were in this series of experiments, determined during the initial period when the maximal flow reduction was still present.

Discussion

The present results indicate that the capacitance vessels in the skeletal muscles are in general far less constricted than the corresponding precapillary resistance vessels when the baroreceptor inhibitory influence on the bulbar vasomotor centre is reduced. This was shown by comparing the reflex responses of the two vascular sections with those obtained by graded electrical stimulations of all regional vasoconstrictor fibres. However, this quantitative difference with regard to the extent of smooth muscle activation in the pre- and postcapillary vessels becomes eliminated when the excitation of the vasomotor centre is intensified. For example, a slight hypoventilation of the animal, which is *per se* not enough to produce any significant constriction of the muscle vessels seems to reinforce the excitation of the vasomotor centre following a diminution of baroreceptor activity and then potentiate the reflex constriction of the postcapillary vessels, so that the reflex engagement of the pre- and postcapillary vessels in the skeletal muscles gradually becomes equally pronounced.

Whether this relatively slight reflex involvement of the veins, as compared with the resistance vessels, in the skeletal muscles is due to an activation of only a fraction of their vasoconstrictor neurons, so that only a limited part of this vascular compartment is engaged, or is due to a lower average discharge rate involving their entire nervous supply is not known for sure at present. However, the apparently extensive degree of divergence-convergence principle with regard to sympathetic innervation of various effector systems (e.g. Hillarp 1960) makes it more likely that a difference in average discharge rate is the main principle of differentiation in this respect.

Since the frequency-response characteristics of the pre- and postcapillary vessels of the skeletal muscles are known from determinations in the present study, it is also possible to deduce approximate figures the difference in constrictor fibre discharge to the pre- and postcapillary vessels of the muscles at different levels of vasomotor centre activity. Such a deduction makes it likely that, even though the venous compartment is probably always to some extent engaged when the baroreceptors are unloaded, the average sympathetic discharge to these vessels is throughout much lower or approximately only half of that to the corresponding precapillary vessel.

The background to this quantitative difference in the reflex engagement of the mentioned series-coupled sections of the muscle vessels is not clear, and a detailed analysis of the possible cause is beyond the scope of the present study. The phenomenon has, however, much in common with the earlier observed quant at d
ference th eg d t reflex adjustme ts of the resistance vessels in various par
coupled rc it (e.g. Lofving 1961 Folkow Johansson and Lofving 1961 The
mentioned studies indicated that the central neurons controlling the muscle
vessels were more easily engaged in e.g. baroreceptor reflexes ha
trolling e.g. the renal or the skin vessels. However when an increased
the motor centre is produced, e.g. by a moderate hypoventilation

id arteries then occluded an intense engagement of the renal resistance vessels also occurs. On the basis of these results it was suggested that the different central neuron pools, controlling the various vascular circuits show variations in their inherent activity level, due to e.g. differences in their excitability to various nervous and humoral influences, in a similar way as the neuron pools of the respiratory centre controlling the different respiratory muscles, exhibit differences in excitability to CO_2, etc. Withdrawal of baroreceptor restraint should then produce the most pronounced activation of those neurons, exhibiting a high "inherent" activity (e.g. those of the muscle vessels) while other vasoconstrictor neurons (e.g. those of the kidneys) will display a high activity upon release from inhibitory impulses first when their excitatory level has been reinforced by e.g. hypoventilation. — It seems reasonable to suggest a similar explanation for the presently observed unequal engagement of skeletal muscle resistance and capacitance vessels, i.e. that the baroreceptor inhibitory impulses affect vasoconstrictor neurons, exhibiting varying levels of activity where the resistance vessel neurons should have a high and the capacitance vessel neurons a low inherent activity.

An alternative explanation for the present findings might be that inhibitory fibres from the baroreceptors are more abundantly distributed to the central neuron pools, controlling the muscle capacitance vessels, than to neurons, controlling the resistance vessels, and that therefore a very drastic diminution of baroreceptor inhibitory in- is required to produce a high discharge rate in the venoconstrictor fibres. possible uneven distribution of the baroreceptor inhibitory fibres is, to judge the present experiments, less likely since even with a supposedly complete unloading of the baroreceptors, the firing rate of the postcapillary vasoconstrictor fibres seemed to be considerable lower than in the fibres, distributed to the resistance essels.

In the intestine as contrasted to the case in skeletal muscle, the constrictor fibres innervating the capacitance vessels, seemed to be activated to the same extent as those to the resistance vessels also when the excitation of the intestinal vasoconstrictor neurons following carotid occlusion was slight or moderate. However as presently shown, and also pointed out by Lofving (1961) the intestinal vascular bed does not seem to be strongly engaged in baroreceptor reflexes, probably due to a low excitatory level of the corresponding vasomotor centre neurons, as discussed above. Thus, flow resistance in the intestine increased only two times in the present series of experiments as compared with 5 to 6 times in skeletal muscle. This different extent of engagement of the two circuits in baroreceptor reflexes suggests that the average vasomotor fibre discharge to the intestine was then considerably lower than that to muscle resistance vessels and was as a matter of fact, rather of the same order of magnitude as the firing rate to the muscle capacitance vessels. — From such comparisons of the extent of activation of various cardio-vascular effectors in baroreceptor reflexes, it might seem more appropriate to ascribe the presently reported, unequal responses of muscle resistance and capacitance vessels to a particularly strong resistance vessel construction rather than to a small capacitance vessel

response. The significance of such an especially marked activation of muscle resistance vessels in baroreceptor reflexes will be further discussed below.

In a recent study using a different technique that involved both muscle and skin vessels of the dog limb Browse, Donald and Shepherd (1966) arrived at the conclusion that the veins were hardly at all involved in the reflex adjustments, produced by carotid occlusion. However, with their technique the interference from the skin vessels, which are on the whole, relatively less engaged in cardiovascular homeostatic reflexes than e.g. the muscle vessels, may be a disturbing element. Undoubtedly their findings point in the same direction as those presently reported, i.e. the muscle veins are generally far less engaged in baroreceptor reflexes than the corresponding resistance vessels. However, the present study indicates that the skeletal muscle veins can indeed be very strongly engaged in baroreceptor reflexes, provided the excitation of the vasomotor centre is intense. The present data also suggest that the unequal reflex engagement of resistance and capacitance vessels in the skeletal muscle, when vasomotor centre excitation is moderate, represents a rather special situation, perhaps special for this particular vascular circuit. In other beds, as e.g. in the intestine, the two vascular compartments seem to be engaged to the same extent in baroreceptor reflex patterns.

It should be pointed out that in the present study venous transmural pressure in the hindquarters and the intestine was kept fairly high in order to minimize the inevitable passive blood mobilization, occurring when venous pressure falls, consequent to a constriction of precapillary vessels (Öberg 1967). The blood mobilization obtained at vasoconstrictor fibre activation can then be more truly related to active venoconstriction. However, despite precautions, a small, passive emptying is unavoidable and the recorded venous responses should, consequently, to a certain extent, be ascribed to a passive phenomenon. — This implies that the active engagement of the veins in baroreceptor reflexes is somewhat overestimated in this study and that, indeed, at low levels of vasomotor centre excitation, venous involvement is very small or perhaps even insignificant.

The functional significance of the mentioned differentiated activation of the vasomotor fibre distributed to the pre and postcapillary vascular sections, respectively, in a skeletal muscle bed, when arterial baroreceptor reflexes are elicited, may be of considerable interest. As mentioned earlier a selective activation of the capacitance vessels, rather than of the resistance vessels would at first sight seem to be the most adequate response to combat arterial hypotension following e.g. blood loss, as then the pressure could be restored without significant restriction of nutritive blood flow to the various organs. However, the reflex constriction of the systemic resistance vessels to carotid sinus hypotension occurs preferentially in the skeletal muscles (Löfving 1961), which tissue during resting conditions seems to tolerate fairly well even rather marked reductions of nutritional blood flow. Therefore, a reflex induced increase of systemic flow resistance by means of a more or less selective constriction of skeletal muscle resistance vessels can be looked upon as a profitable compensatory mechanism in arterial hypotension, although this factor alone does not produce

HILLARP N A Peripheral autonomic mechanisms. *Handbook J Physiol.* 1960, Sect. 1 Vol. 2. 979—1006

LÖFVING, B., Cardiovascular adjustments induced from the rostral cingulate gyrus. *Acta physiol. scand.* 1961 53 Suppl. 184 1—82

LUNDGREN, O J LUNDVALL and S. MELLANDER, Range of sympathetic discharge and reflex vascular adjustments in skeletal muscle during hemorrhagic hypotension. *Acta physiol. scand.* 1964 62 380—390

MELLANDER, S Comparative studies on the adrenergic neurohumoral control of resistance and vascular resistance. *Circulat Res* 1961 9 1327—1335

MELLANDER, S Comparative studies on the adrenergic neurohumoral control of resistance and capacitance blood vessels in the cat. *Acta physiol scand* 1960 50 Suppl. 176 1—86.

ÖBERG, B., Effects of cardiovascular reflexes on net capillary fluid transfer *Acta physiol. scand* 1964 62 Suppl. 229 1—98.

ÖBERG, B The relationship between active constriction and passive recoil of the veins at various distending pressures. *Acta physiol. scand* 1967 71 233—247

OPITZ, E. and D H SMYTH Nierendurchblutung bei Reizung des Carotis-Sinus. *Arch. ges. Physiol.* 1937 238 633—637

POLOSA, C. and G ROSSI, Cardiac output and peripheral blood flow during occlusion of carotid arteries. *Am J Physiol. 1961 200* 1185—1190

Acta physiol. scand. 1968. 72. 533—534

From the Neurosurgical Research Laboratory and the Neurosurgical Service A, Lasarettet, Lund, and from the Department of Neurosurgery University of Lund Sweden

Tissue NADH Levels in the Rat Brain during Pronounced Hyperventilation

By

Lars Granholm Ludmilla Lukjanova and Bo K. Siesjö

Since pronounced hyperventilation leads to a lower [illegible] of the cerebral blood flow associated with EEG changes, a decrease in th[illegible] tissue O[illegible] tension, and an increased anaerobic glycolysis, it has been assumed to [illegible] a [illegible] hypoxia (Kety and Schmidt 1946, Sugioka and Davis 1960 Alexander et al. 196[illegible]) We have recently reported that pronounced hyperventilation [illegible] leads [illegible] increased lactate/pyruvate ratio in both the cerebrospinal fluid and [illegible] the brain cortex (Granholm and Siesjö 1968 a) This is indirect evidence of an increased NADH/NAD [illegible] in the tissue since the lactate/pyruvate system is supposed to be [illegible] near-equilibrium with the NAD system. However the lactate/pyruvate [illegible] were accompanied by normal, or near normal, ATP/ADP and P-Creatine/Creatine ratios (Granholm and Siesjö 1968 b) whence the interpretation of the la[illegible] changes [illegible] made difficult.

[illegible] order to elucidate tissue redox [illegible] in [illegible] brain during pronounced hyperventilation we have recorded [illegible] NADH [illegible] brain cortex of rats, using the microfluorometric method described by Chance et al. 1962 The rats were anaesthetized with phenobarbital (50 mg/kg [illegible]) [illegible] and ventilated artificially with [illegible] [illegible] A femoral artery [illegible] for blood sampling and subsequent measurements of pO_2, pCO_2 and pH A small [illegible] made in the parietal region. The NADH measurements [illegible] through the [illegible] dura, using the excitation energy from [illegible] kW mercury ar[illegible] 366 mμ prima[illegible] filter and [illegible] secondary filters (Wratten type 2 C [illegible] 47 A The optical field [illegible] was around 3[illegible] was chosen so that no larger vessels were [illegible] The fluorescence changes [illegible] obtained from [illegible] comparison [illegible] the fluorescence [illegible] from [illegible] normal [illegible] to [illegible] maximum change at the cessation of [illegible] shown in [illegible] been here

Fig. 1 illustrates the fluorescence changes during two successive hyperventilation [illegible] during which $PaCO_2$ was decreased from 33.5 mm Hg to 12—13 mm Hg. The [illegible] sampl[illegible] [illegible] indicated by triangles It is seen that the fluorescence [illegible] pl[illegible] [illegible] 3—4 mi[illegible] after the start of hyperventilation, and th[illegible] [illegible] followed by [illegible] distinct overshoot below the fluorescence [illegible] We ha[illegible] carried ou[illegible] 28 h[illegible] hyperventilations [illegible] 14 experiments [illegible]

[illegible] from Institute of Biophysics, Academy of Scienc[illegible]



Fig 1 Microfluorometric recording of tissue NADH levels in rat brain cortex. The time scale proceeds from right to left. Increases in fluorescence are recorded as upward deflections. The animal was ventilated with air until the end of the experiment when nitrogen was given. Note increase in fluorescence during 2 consecutive hyperventilations, and overshoot below baseline after the first hyperventilation.

ventilation to PaCO values of 11 5—12.5 mm Hg led to an increased fluorescence, which in average amounted to 25 of the value recorded at the cessation of cardiac activity during nitrogen breathing. In at least half of the hyperventilations an over shoot was seen after the resumption of normal ventilation. This could reflect a reactive hyperemia following the period of decreased blood flow. Administration of CO_2 during continuous hyperventilation invariably led to a decrease in fluorescence to base line levels, whereas 100 O had a much smaller effect.

It is concluded that pronounced hyperventilation leads to an increased NADH NAD ratio in the tissue probably caused by the decreased blood flow. Further studies will be needed to interpret the relation between these changes and the concomitant changes in other metabolites involved in the energy metabolism of the tissue.

This study was supported by the Swedish Medical Research Council Project B67 14X 263-03) and by Ollie and Elof Ericsson Stiftelse.

References

ALEXANDER, S C P J C H W M T C S M REIV and R W M LES, Cerebral carbohydrate metabolism during hypocarbia in man. Anesthesiology 1965 26 624–632

CHANCE, B P COHEN F JOBSIS and B SCHOENER Intracellular oxidation reduction states in vivo. Science 1962 137 499 508

GRANHOLM L. and B K SIESJÖ Signs of cerebral hypoxia in hyperventilation. Experientia (Basel) 1968 in press

GRANHOLM L and B K SIESJÖ The effect of hypercapnia and hypocapnia upon the cerebrospinal fluid lactate and pyruvate concentrations and upon the lactate pyruvate ATP ADP phosphocreatine and creatine concentrations of rat brain tissue 1968 To be published

KETY S. S and C F SCHMIDT The effects of active and passive hyperventilation on cerebral blood flow cerebral oxygen consumption cardiac output and blood pressure of normal young men. J Clin Invest 1946 25 107 119

SUGIOKA, K and D A DAVIS Hyperventilation with oxygen a possible cause of cerebral hypoxia. Anesthesiology 1960 21 135 143

Acta physiol. scand. 1968. 72. 535—536

From the Institute of Physiology and Medical Biophysics, University of Uppsala, Sweden

Internal Perfusion of Crayfish Axons

By

B. Gunnar Wallin

Since its introduction (Oikawa *et al.* 1961 Baker Hodgkin and Shaw 1961) internal perfusion of axons has been recognized as a most valuable tool for investigating the properties of the nerve membrane. So far however only fairly large squid axons have been successfully used for internal perfusion and as squids are available only for a short period of the year in very limited areas, internal perfusion of axons has been possible only at a few selected places. It is the purpose of this communication to demonstrate that internal perfusion is feasible in the much smaller axons from crayfish, an animal readily obtained almost anywhere in the world and which conveniently can be kept in captivity for many months.

The ventral nerve cord from the crayfish *Procambarus clarki* was dissected free in its entire length and mounted in a Perspex chamber filled with van Harreveld's solution of the following composition (in mM) NaCl 205 KCl 5.4 $CaCl_2$ 13.5 $MgCl_2$ 2.6, $NaHCO_3$ 2.3 One medial giant axon, usually of a diameter of 125—175 μ was isolated between ganglia 2—5 A hole was cut in the axon near ganglion 6 and an L-shaped double pipette was inserted lengthwise into the axon. One pipette was a potential recording micro-electrode connected to a unity gain cathode follower The other pipette, the tip of which was 50—80 μ in diameter and situated about 3 mm behind the tip of the potential electrode, was the perfusion pipette and was connected to a syringe via an oil filled plastic tubing. The two pipettes were glued together with insulating laquer When the tip of the perfusion pipette had entered the axon a ligature was tied around the pipette shanks. The perfusion solution, e.g. KF 230 mM buffered with Sörensen's phosphate buffer to pH 7.3 was enclosed in the perfusion pipette by a little drop of silicone oil. This oil served as indicator of the initial velocity of the fluid movement. After a drainage hole was cut at ganglion 2 the perfusion was started by adjusting the syringe at a suitable level giving enough perfusion pressure As crayfish axoplasm is quite fluid little pressure is needed to replace the normal axoplasm with perfusion fluid. Furthermore if too much pressure is applied the action potential is immediately abolished irreversibly A schematic picture of the setup is given in Fig. 1

With this system the axon could be perfused for close to one hour with maintained resting and action potential. Fig. 2 shows a comparison between an action potential

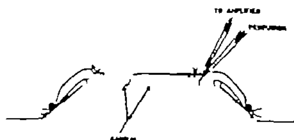
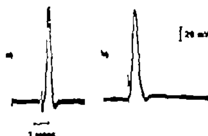


Fig 1 Schematic pictur of the experimental set up. Perfusion flow is from right to left as indicated by arrows.



F g. 2. Comparison of action potentials from) axon with normal axoplasm and b) same axon after 12 min internal perfusion with 250 mM K. The anions a mixture of 4/5 fluoride and 1/5 propionate.

the normal axon and after perfusion for 12 minutes. Different anions were ed in the perfusion fluid and although no systematic study has yet been made the results seem to agree with the finding of Tasaki, Singer and Takenaka (1965) in that fluoride is a preferred anion in contrast with for example chloride which very rapidly caused failure of the actio n potential and a steady decline of the resting potential.

This work was supported by US-Public H alth service grant NIH NB 037-04-05 and the Swedish Medical Research Council grant B 68-14 -629-04

References

BAKER, P F A L HODGKIN and T I SHAW Replacement of the protoplasm of giant nerv fibre with artificial solutions. Natur (Lond.) 1961 190 885—887

OIKAWA, T C S SPYROPOULOS I TASAKI and T TEORELL, Methods for perfusing the giant axon of Loligo Pealii. Act physiol. sc nd 1961 52 195—196.

TASAKI, I., I SINGER and T TAKENAKA, Effects of internal and external ionic env ronment on excitability of squid giant axon. J gen. Physiol. 1965. 48 1095—1123